

**STRATEGIES TO MITIGATE CHEMOTHERAPY-RELATED
CARDIOTOXICITIES IN TRIPLE-NEGATIVE BREAST CANCER**

BY

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**I dedicate this work to my mother, Cristina Mosquea, and my grandparents,
Andrea Navarro and Enrique Mosquea, to whom I owe everything.**

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LIST OF ABBREVIATIONS

ACEIs	Angiotensin-converting enzyme inhibitors
Als	Aromatase inhibitors
Akt	Protein kinase B
ALS	Amyotrophic lateral sclerosis
AMPK	AMP-activated protein kinase
AR	Androgen receptor
ARBs	Angiotensin receptor blockers
AREs	Antioxidant response elements
ASK1	Apoptosis signal-regulating kinase 1
ATF4	Activating Transcription Factor 4
ATF6	Activating transcription factor 6
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and rad3-related protein
BC	Breast cancer
BCL2	B-cell lymphoma 2 - Apoptosis regulator
BCL-W	B-cell lymphoma W - Apoptosis regulator
BCL-XL	B-cell lymphoma XL - Apoptosis regulator
BL-1	Basal-like subtypes 1
BL-2	Basal-like subtypes 1
BNP	B-type natriuretic peptide

BRCA1	Breast Cancer gene 1
BRCA2	Breast Cancer gene 2
bZIP	Basic leucine zipper
CAFs	Cancer-associated fibroblasts
CD47	Cluster of Differentiation 47 or integrin-associated protein
CHF	Congestive heart failure
CHK1/2	Checkpoint kinases 1/2
CHOP	C/EBP homologous protein
CK	Cytokeratin
CMF	Cyclophosphamide – Methotrexate – 5 Fluorouracil
CTLA4	Cytotoxic T lymphocyte-associated pattern 4
Cu	Copper
CV	Cardiovascular
CVD	Cardiovascular disease
DDI3	damage-inducible transcript 3 or CHOP
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
ECM	Extracellular matrix
EF	Ejection fraction
EGFR	Epidermal growth factor receptor
eIF2 α	Eukaryotic translation initiation factor 2 α -subunit
eNOS	Endothelial nitric oxide synthase
ER	Estrogen receptor

ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum-associated protein degradation
ERBB2	Erythroblastic leukemia viral oncogene homolog 2
ERO1 α	Endoplasmic reticulum oxidoreduction 1 α
FDA	Food and Drug Administration
Fe	Iron
FGF	Fibroblast growth factor
FS	Fractional shortening
GRP78	Glucose-regulated protein 78
HER-2	Human epidermal growth factor 2
HF	Heart failure
HFpEF	Heart failure with preserved ejection fraction
HF _r EF	Heart failure with reduced ejection fraction
HGF	Hepatocyte growth factor
Hmox1	Heme oxygenase-1
HSF-1	Heat shock factor 1
HSP-25	Heat shock protein
I/R	Ischemia/reperfusion
ICIs	Immune checkpoint inhibitors
IHC	Immunohistochemistry
IL-1 β	Interleukin 1 β
IL-6	Interleukin 6
IL-8	interleukin 8

iNOS	Inducible nitric oxide synthase
IP	Intraperitoneal
IP3R	Inositol 1,4,5-trisphosphate receptors
IRE1	Inositol-requiring enzyme 1
IRE1M	Inositol-requiring enzyme 1 morpholino inhibits IRE1 translation
IV	Intravenous
JNK	c-JUN N-terminal kinase
KEAP1	Kelch-like ECH-associated protein 1
Ki-67	Cellular marker for proliferation
LAMP3	Associated membrane protein 3 to lysosomes
LV	Left ventricle
LVEF	Left ventricle ejection fraction
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MMP-1	Matrix metalloprotease 1
MMP-2	Matrix metalloprotease 2
MMP-9	Matrix metalloprotease 9
MRIs	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NADPH	Adenine dinucleotide phosphate
NFkB	Nuclear factor kappa B
NLD	N-terminal luminal domain

nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthases
NOX	NADPH Oxidases
NOX2	NADPH Oxidase 2
Nqo1	NADPH quinone oxidase-1
Nrf2	Nuclear factor erythroid 2-related factor 2
NT	Nitrotyrosine
O ₂ ^{•-}	Superoxide
OCR	Oxygen consumption rate
PAK2	Serine/threonine protein kinase 2
pCR	Pathologic complete response
PD-1	Programmed cell death 1
PDGF	Platelet-derived growth factor
PDI	Protein disulfide isomerase
PD-L1	Programmed cell death 1 ligand
PERK	PKR-like endoplasmic reticulum kinase
p-JNK	c-Jun N-terminal kinase, phosphorylated
PR	Progesterone receptor
RAS	Renin-angiotensin system
RIDD	Regulated IRE1 α -dependent decay
RNase	endoribonuclease domain
RNS	Reactive nitrogen species

ROS	Reactive oxygen species
RT	Radiation therapy
RyR	Ryanodine receptors
S1P	Sphingosine-1-phosphate
S2P	Sphingosine-2-phosphate
SAD	Standard American diet
SMAD	Small Mothers against decapentaplegic
SMAD2/3	Small mothers Against decapentaplegic 2/3
SMAD4	Small mothers Against decapentaplegic 4
SS	Signal sequence
Sxn1	Sulfiredoxin-1
TGF- β	Transforming growth factor-beta
TGF- β 1	Transforming growth factor beta 1
TILs	Tumor-infiltrating lymphocytes
TNBC	Triple-negative breast cancer
TNF- α	Tumor necrosis factor-alpha
Top-II α	Topoisomerase-II α
Top-MT	Genetic ablation of mitochondrial topoisomerase
TRAF2	Tumor necrosis factor receptor-associated factor 2
TRIB3	Through regulation of homologous tribologist 3
ULK1	Autophagy activating kinase similar to unc-51
UPR	Unfolded Protein Response
VEGF	Vascular endothelial growth factor

XBP1-S

X-box binding protein 1 spliced

XBP1-U

X-box binding protein 1 unspliced spliced.

ABSTRACT

STRATEGIES TO MITIGATE CHEMOTHERAPY-RELATED CARDIOTOXICITIES IN TRIPLE-NEGATIVE BREAST CANCER

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Triple-negative breast cancer (TNBC) is one of the most highly aggressive breast cancer types that predominantly affects young and minority women. TNBC patients are more likely to receive cytotoxic chemotherapy regimens since they have limited targeted options. This results in severe side effects resulting in chronic cardiac dysfunction. Currently, there are limited options for the treatment of cardiotoxicity that do not interfere with the anti-cancer action of chemotherapy drugs. Consequently, there is an urgent need to find new strategies that decrease the off-target effects of chemotherapy without compromising oncologic efficacy. Anthracycline (DOX) is a very effective chemotherapeutic agent; however, it is the most harmful toxic agent used to treat different types of cancer, including breast cancer.

Another issue compounding the risk of developing cancer and chemotherapy-related toxicities is obesity. Obesity is associated with worse overall survival in women with TNBC. Inositol-requiring enzyme-1 (IRE1) is an arm of the

unfolded protein response (UPR) pathway that plays a crucial role in tumor development and activates MAPK kinase and NF- κ B, which contribute to inflammation in obesity. Additionally, IRE1 is a conserved regulator of the human Nrf2 antioxidant response. It has been shown that IRE1/XBP1 protein levels are upregulated in TNBC. Preliminary data suggested that targeting IRE1 in combination with Doxorubicin (DOX) enhances chemotherapy responsiveness in the 4T1 breast cancer model and reduces metastasis. Moreover, inhibiting IRE1 prevents long-term DOX-mediated cardiac damage by reducing fibrosis.

Therefore, to determine how targeting the IRE1 arm of the UPR pathway can potentially prevent the onset of cardiac dysfunction in obese and non-obese cancer patients undergoing chemotherapeutic treatments, female BALB/c mice were placed on control (low fat) and Western (high fat) diets. Then, mice were injected with 4T1-luciferase murine TNBC cells into mammary gland tissue. Mice were treated with doxorubicin (DOX) with or without IRE1 morpholino. Cardiac function was measured by ultrasound at baseline and the end of the study. Our data demonstrate that targeting IRE1 in TNBC tumors in Western diet-fed mice reduces tumor growth and increases oxidative stress. Moreover, our data suggest that IRE1 blockade may prevent metastasis formation in Western diet-fed mice. Furthermore, targeting IRE1 prevents chemotherapy-mediated cardiotoxicity in control and Western diet. Also, targeting IRE1 reduces cardiac injury markers in the control diet and fibrosis in the control and Western diet. Furthermore, IRE1 blockade regulates mitochondrial oxygen consumption as an acute response to cardiac damage. Overall results suggest that systemic suppression of IRE1

protected cardiac tissue in mice treated with doxorubicin while enhancing anthracycline-mediated tumor killing. Throughout this dissertation, we uncovered that targeting the IRE1 signaling pathway is a novel strategy to improve cytotoxic chemotherapy of triple-negative breast cancer, bringing a novel approach to identify better treatment options for TNBC patients to improve overall survival and quality of life.

CHAPTER I

Introduction

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Breast Cancer Statistics Overview

Cancer is a major global health problem that affects females and males of any age. According to the American Cancer Society, cancer is the second leading cause of death worldwide (after cardiovascular diseases). Cancer causes approximately 1 in 6 deaths worldwide. In 2020, 19.3 million new cancer cases were diagnosed, with approximately 10.0 million cancer deaths worldwide (these worldwide incidence and mortality data were collected from 36 cancers in 18 countries) [1]. The same study demonstrated that breast cancer is the most commonly diagnosed cancer in females, with an estimated 2.3 million new cases (11.7% of total cases) and a total of 684,996 (6.9% of total cases) deaths worldwide. In the United States of America (USA), an estimated 276,480 new female breast cancer cases were diagnosed, with more than 42,000 deaths in 2020 [2]. In 2021, there were an estimated 281,550 new female cases of breast cancer and 43,600 deaths in the USA [3]. Also, in 2022, around 287,850 new female breast cancer cases were diagnosed, with 43,250 deaths in the USA [4]. Furthermore, the American Cancer Society estimated that 297,790 new female breast cancer cases are projected to occur in the USA in 2023 [5]. Based on the number of breast cancers estimated to occur in the USA in 2023, 10,730 female cases of breast cancer are predicted to occur in North Carolina [5]. Also, Breast cancer is the second highest killer of all cancer types in women, second only to lung and bronchial cancer, with more than 43,000 predictable deaths in the United States in 2023 [5].

Breast cancer incidence rates have increased over the years due to hormonal risk factors, lifestyle risk factors, genetic mutations, and other risk factors [6] [7] [8]. Indeed, according to the American Cancer Society, female breast cancer mortality has decreased by 43% (drawn in 1989) because of earlier diagnosis and improvements in treatment [2] [3] [4] [5]. On the other hand, it is expected that due to the impact of the pandemic (COVID-19) on cancer detection and treatment, mortality will increase [9]. According to the National Cancer Institute, it is estimated that mortality of breast cancer will increase by approximately 1% over the next 10 years, the equivalent of approximately 10,000 additional deaths due to the impact of the pandemic on detection and treatment. [10].

Breast Cancer Subtypes

The breasts are mainly made up of adipose tissue [11]. Both females and males have breasts, but females have more glandular tissue than males [12]. Moreover, female breasts contain more than 12 lobes divided into smaller lobules connected via milk ducts [11]. The adipose tissue of the breast is rich in nerves, blood vessels, veins, lymph nodes, and lymph vessels (**Figure 1**) [12] [13].

Breast Cancer (BC) is a miscellaneous and complex disease with various clinical and pathological features that starts when cells grow out of control. There are different types of breast cancer classified by location, size, grade, node involvement, metastases, and genetic molecular expression profiling of the receptor status [14] [15] [16]. Based on breast cancer classification of molecular

profiling of the receptor status such as estrogen receptor (ER), progesterone receptor (PR), and the amplification of the human epidermal growth factor 2 (HER-2/neu, also known as ERBB2) receptor, allows the division of breast cancer cases into five primary subcategories **(Figure 2)** [17] [18] [19].

1. **Luminal A and B:** Luminal breast cancer is divided into two types: luminal A and luminal B. Luminal A and B express estrogen receptors (ER) that play an important role in tumorigenesis, proliferation, and cell survival. Luminal A and B tumors represent 60-70% of all breast cancer cases. These tumor types are sensitive to hormonal therapies such as aromatase inhibitors (AIs) or tamoxifen, but adverse side effects may limit their use in patients [20]. There are essential characteristics of Luminal A and Luminal B. Luminal A breast cancer is estrogen receptor-positive, progesterone receptor-positive, and the human epidermal growth factor receptor-2 (HER2)-negative [21] [22]. Luminal A tumors typically express a higher ER-regulated gene expression and have a lower breast cancer cell proliferation index (low levels of the Ki-67 proliferation protein) compared to luminal B breast cancer [23]. Additionally, luminal A tumors tend to have the best prognosis with high survival and low breast cancer recurrence rates [24] [25] [26]. On the other hand, luminal B tumor is estrogen receptor-positive and may be progesterone receptor-positive or progesterone receptor-negative. Also, luminal B tumors may express an elevated level of HER2 and tend to grow faster than luminal A tumors. Additionally, luminal B breast cancer has a poorer prognosis than luminal A tumors [26] [27].

2. **HER2 type:** Human epidermal growth factor receptor (HER)-2 is ER-negative, PR-negative, and characterized by the over-expression of HER2 or ERBB2 (erythroblastic leukemia viral oncogene homolog 2) [15] that account for 15-25% of all breast cancer cases. These types of breast cancer grow faster and have a poorer prognosis than luminal tumors. HER2-amplified tumors respond to targeted therapies such as trastuzumab, Herceptin (toxicities limit their use in the clinic), a monoclonal antibody that blocks the HER2 receptor, or lapatinib (Tykerb), which is a dual tyrosine kinase inhibitor that blocks signaling through HER2 [20].
3. **Basal-like or Triple-negative breast cancer** [reviewed in reference [28] [29]. Basal-like or Triple-negative breast cancer (TNBC) is ER-negative, PR-negative, and HER2-negative. This type of breast cancer accounts for 10-20% of breast cancer cases. TNBC is more common in women under 40 years of age, minority populations, and women with a *BRCA1* mutation. This type of breast cancer has a poor prognosis and is very aggressive. TNBC can be characterized by the expression of markers for cytokeratin 5/6 and the epidermal growth factor receptor (EGFR) [15]. There are no targeted therapeutic options for these types of breast cancer patients, and they are limited to cytotoxic chemotherapeutics such as adjuvant and neo-adjuvant treatment [30] [31] [32].
4. **Normal breast-like:** This type of breast cancer accounts for 5-10% of all breast cancer cases and has a profile of genes expressed by adipose tissue. Normal breast-like tumors are similar to the luminal A subtype in

terms of hormone status (ER and PR-positive, HER2-negative) and outcomes [33] [34].

Additionally, disparities in breast cancer outcomes are due to genetic or social/health system determinants of race. It is estimated that 5–10% of breast cancers are the result of inherited gene mutation [35]. These gene mutations are most commonly found in genes named Breast Cancer gene 1 and Breast Cancer gene 2 (*BRCA1 and BRCA2*) associated with greater breast cancer rates. It is estimated that the risk of developing breast cancer is 55-72% for *BRCA1* and 45-69% for *BRCA2* mutation carriers [36]. On the other hand, the statistical evidence has shown that breast cancer incidence rates are highest among white patients, closely followed by black patients. However, black women have the highest mortality rate from breast cancer, which predominantly affects younger women, such as those under 50 years of age. [35] [37].

Moreover, the treatments for breast cancer will depend on the subtypes of breast cancer, the stage, and the presence of metastasis (**Table 1**). Early treatment for breast cancer usually involves breast conservation surgery, such as a lumpectomy or partial mastectomy, where the primary tumor is removed and the cancer cells in the axillary nodes are examined. Also, a total mastectomy may be performed where the breast is removed with the cancer or where the lymph nodes and chest wall muscles are removed along with the cancer [20]. After surgery, radiation therapy may be suggested to kill remaining cancer cells and reduce the risk of local recurrence and is recommended for patients undergoing breast conservation surgery. Treatment for breast cancer

may also include adjuvant and neoadjuvant therapies, given before or after surgery with or without radiation (recommended when the patient is at risk for spread disease) [38]. Systemic or regional chemotherapy agents for the adjuvant and neoadjuvant treatment of breast cancer include anthracyclines (doxorubicin and epirubicin), taxanes (paclitaxel and docetaxel), 5-fluorouracil (5-FU) or capecitabine and cyclophosphamide, and agents platinum-based (carboplatin). These chemotherapeutic agents have a mechanism of action that interferes with the survival of cancer cells and reduces the risk of breast cancer recurrence [39]. As mentioned, depending on the breast cancer status, patients may undergo specific treatment; patients with overexpressed HER2 can be treated with anti-HER2 antibodies. Also, patients with hormone-sensitive tumors (ER positive, PR positive) can be treated with hormonal therapies to prevent recurrence and spread of breast cancer cells [40] [41] [42] [43] [44].

Figure 2 shows that these five groups represent the main subcategories of breast cancer classified by molecular profiling analysis. Accordingly, when breast cancer is diagnosed, several clinical studies (tests) are performed to determine the grade and the stage of breast cancer, and then treatment options will be performed. The majority of this thesis work involves the investigation of novel strategies to lessen the toxic side effects of cytotoxic chemotherapy regimens associated with cardiac dysfunction in obese and non-obese triple-negative breast cancer patients. Furthermore, it involves the investigation of possible viable targeted therapy options for triple-negative breast cancer. This subgroup of breast cancer is described in more detail in the following sections.

Triple Negative Breast Cancer (TNBC)

Triple-negative breast cancer (TNBC) is one of the most aggressive subtypes of breast cancer that tends to grow and spread more quickly than other types of breast cancer. It is estimated that out of the worldwide breast cancer burden, approximately 170,000 cases are TNBC [45] [46] [47]. According to the American Cancer Society, this type of breast cancer accounts for 10-20% of all breast cancer cases. Moreover, TNBC lacks the molecular expression of all three receptors, such as estrogen receptor (ER), progesterone receptor (PR), and not the human epidermal growth factor receptor-2 (HER2) gene amplified (making it ER-, PR-, and HER2-negative) [29]. Due to the lack of these receptors, it is more challenging to treat this type of cancer with hormone therapy or targeted therapy. Therefore, TNBC patients are more likely to be treated with toxic treatments, such as chemotherapy-based regimens. Consequently, these patients are at high risk of developing life-threatening chronic diseases, mainly in the cardiovascular system [31] [48] [49]. Furthermore, studies have shown that the heterogeneity of TNBC (basal-like subtypes (BL-1, BL-2), mesenchymal-like subtype, luminal androgen receptor subtype, and immunomodulatory subtype) results in variable responses to treatments, limiting treatment options [50].

TNBC is a terrible disease whose development is subject to specific risk factors such as age, BRCA1 gene mutation, and ethnicity [35] [37]. Also, premenopausal women and those under the age of 40 have an increased risk of developing TNBC when compared to older, postmenopausal women. It has

been observed that more than 50% of diagnosed breast cancers associated with an inherited mutation in *BRCA1* are TNBC and express a high amount of basal-like cytokeratins (CK5,14,17), as well as P-Cadherin and HER1/EGFR [45] [51] [52]. Additionally, TNBC is more common in African American and Hispanic women and women from areas of lower socioeconomic status [35] [37].

Triple Negative Breast Cancer Symptoms and Diagnosis

The symptoms of triple-negative breast cancer (TNBC) are similar to the other breast cancer subtypes. According to the American Cancer Society, breast cancer symptoms are a new lump or mass (even though most breast lumps are not cancer) or abnormality on a routine mammogram and ultrasound. Also, other possible symptoms of breast cancer that should be addressed are redness of the skin of the breast, swelling, and pain in the breast (especially if it is more concentrated on one side than the other), bleeding from the nipple, nipple inversion (your nipple starts pointing inward instead of outward), swelling or lumps in the underarm areas, or any other significant change from your usual pattern.

In general, the methods for diagnosing breast cancer are the same as for detecting TNBC. Still, as I mentioned, TNBC has specific characteristics that distinguish it from other types of breast cancer in terms of molecular profile, age, gene mutation, *BRCA1*, and ethnicity. After some suspicious symptoms,

such as a lump in the breast or an abnormal mammogram, several imaging tests are performed to find out specific characteristics to detect the cancer. The imaging tests used include 3D mammograms, ultrasounds, and MRIs. After imaging tests, a breast biopsy is used to confirm the findings (breast cancer). It has been shown by mammography and ultrasound that the morphological characteristics of triple-negative breast cancer include a lobulated mass with less attenuating posterior echoes, some vascularization, and low elasticity [53].

Triple Negative Breast Cancer Treatment

Scientists and doctors are making great efforts to understand what causes triple-negative breast cancers to grow and to develop new treatments since hormone receptors (ER-negative, PR-negative) and human epidermal growth factor receptor 2. (HER2-negative) are not present. Consequently, treating TNBC is challenging because it will depend on the patient's conditions, tumor stage, and the presence of metastasis. Here are some of the treatment options for patients with TNBC:

1. Surgery – Lumpectomy or mastectomy can be done in TNBC patients.

In stages I-III of TNBC, surgery can be considered the first option, followed by radiation, chemotherapy, and immunotherapy in some cases [54] [55]. A lumpectomy is for tumors smaller than 4 centimeters, requires subsequent radiation, and clear margins indicate that all signs of cancer have been removed [56]. Mastectomy is for more extensive

tumors (more than 4 cm) or tumors that are difficult to operate on. A double mastectomy may be most beneficial for women under age 50, followed by radiation for large tumors or those with four or more lymph nodes involved [57].

2. **Radiation Therapy** – Radiation is the energy propagating through space/matter as waves/packets. Radiation therapy (RT) is used to treat TNBC (and cancer in general) specifically after surgery, either lumpectomy or mastectomy, and when metastases occur in the bones or brain [58] [59]. RT is considered an essential option for optimal long-term outcomes for cancer patients, where high-energy rays or particles can directly hit the DNA, causing damage to cancer cells or contributing to the formation of radicals using water that leads to the generation of ions that indirectly cause DNA damage.
3. **Chemotherapy** - Chemotherapy is systemic, targeting the entire body and killing local and distant cancer cells. It has demonstrated that chemotherapy is very effective against invasive and metastatic cancers such as TNBC. Thus, chemotherapy can work in multiple ways, such as stopping cancer cell proliferation by altering the structure of the DNA of those cells, interfering with the creation of new cells, which blocks the formation of new DNA, or stopping the mitotic process (the point at which a cancer cell divides into two cells). Chemotherapy can be done before or after surgery:

- a. **Neoadjuvant chemotherapy** - Neoadjuvant chemotherapy is administered before surgery and is generally used for cases where surgery is complex with large tumors, specifically those tumors that have spread or metastasized (to the lungs, brain, and liver) that would require multiple surgeries or those that are difficult for a surgeon to reach safely or effectively. Neoadjuvant chemotherapy can use a single agent or a combination of chemotherapeutic drugs. Research showed that 1,118 breast cancer patients who received neoadjuvant chemotherapy at MD Anderson Cancer Center for stage I-III breast cancer, 255 patients, had TNBC. Of those patients with TNBC, 22% had a complete pathologic response compared with three-year overall survival rates [60]. Also, other research concluded that neoadjuvant chemotherapy showed a higher pathologic complete response (pCR) in the TNBC setting than in non-TNBC [61]. Anthracycline and taxane regimens are the standard regimens for treating patients with breast cancer, especially TNBC. However, the optimal chemotherapeutic regimen in neoadjuvant treatment for TNBC is still debated [62] [63]. It has been shown that there are differences in the efficacy of neoadjuvant regimens used for TNBC. For example, the basal-like (TNBC subtype) responds better to taxane-based therapy and shows worse response outcomes to anthracycline-based treatment compared to other

breast cancer subtypes [64]. TNBC with *BRCA1* mutation are more sensitive to platinum agents [65].

- b. **Adjuvant chemotherapy**—Adjuvant chemotherapy is performed after surgery for tumors smaller than 4 cm, especially those with minimal local spread. Surgery can effectively remove the cancerous tumor, and adjuvant chemotherapy will kill any cancer cells that may remain. Adjuvant chemotherapy is more effective in hormone receptors status at early stages.

The most common chemotherapy agents for TNBC combinations are Adriamycin (Doxorubicin), Cytosan, and Taxane, although recently, a combination of Cytosan-taxane without Adriamycin has been used. All of these agents have side effects because they are essentially toxins that attack the cancer but affect the rest of the body. Examples of chemotherapeutic agents used in neoadjuvant and adjuvant chemotherapy are found in **Table 1**.

- 4. **Immunotherapy** - Tumors have many mechanisms for their growth and progression. Understanding why some cancers progress while others do not by finding strategies to evade anti-tumor immunosurveillance is a longstanding challenge in immunology [66]. The hallmarks of cancer exemplify that tumors have several mechanisms that allow their progression and contribute to metastasis by avoiding immune destruction and deregulating cellular energetics. It has been demonstrated that a critical point in cancer progression is evading

recognition by the immune system. Cancer cells accomplish this by stimulating various immune checkpoint proteins on effector T-cells such as cytotoxic T lymphocyte-associated pattern 4 (CTLA4), programmed cell death 1 (PD-1), and PD-1 ligand (PD-L1) [67]. For several cancers, the Food and Drug Administration (FDA) approved monoclonal antibodies against the immune checkpoint inhibitors (ICIs) that block immunosuppressive receptors, CTLA-4 (ipilimumab), PD-L1 (atezolizumab), and PD-1 (pembrolizumab) to improve the cytotoxicity of tumor-infiltrating lymphocytes (TILs) [68]. These therapies function as an ON and OFF switch.

Immunotherapy for TNBC has accelerated research into immunoncology drugs for better response, outcomes, and survival rates for this terrible disease. TNBC is characterized by PD-L1 expressed on tumor and immune cells and TILs associated with a better response to immunotherapy [69] [70]. The FDA approved the combination of Tecentriq (atezolizumab) and Abraxane (nab-paclitaxel) for patients with metastatic TNBC (highly expressed PD-L1). Additionally, to receive this combination of treatments, patients with metastatic TNBC must have received at least 1 year of adjuvant or neoadjuvant taxane [29] [71]. Recently, FDA approved pembrolizumab (Keytruda) for high-risk, early-stage, triple-negative breast cancer in combination with neoadjuvant chemotherapy agents followed by adjuvant treatment. Research showed that immune-stimulating therapies act synergistically when

combined with chemotherapy in the early and advanced stages of the disease [72] [73].

New upcoming target treatments for TNBC (under active clinical evaluation) are reviewed in [29].

1. Poly (ADP Ribose) Polymerase inhibitors (i.e., iniparib, Olaparib) [74]
2. Vascular Endothelial Growth Factor (VEGF) inhibitors (i.e., bevacizumab) [75]
3. Epidermal growth factor receptor (EGFR) inhibitors (i.e., afatinib and dasatinib) [76] [77]
4. Fibroblast Growth Factor (FGF) inhibitors (Infigratinib) [78] [79]
5. Androgen Receptor (AR) such as bicalutamide and palbociclib [80] [81]
6. Etoposides (i.e., ixabepilone) [82] [83]
7. The Mammalian target of rapamycin (mTOR) inhibitors (i.e., everolimus) [84]
8. Other promising therapeutic targets such as DNA damage-induced cell cycle arrest, DNA damage checkpoint kinases including CHK1/2 (checkpoint kinase 1/2), ATR (ataxia telangiectasia and rad3-related protein), and ATM (ataxia telangiectasia mutated) [29] [85] [86] [87]

Doxorubicin (DOX) review in [88].

Doxorubicin (DOX) is a very effective chemotherapy agent used since the 1960s in different types of cancer, including breast cancer, Kaposi's sarcoma, acute lymphocytic leukemia, lymphoma, and bladder cancer [89, 90]. DOX is isolated from a bacteria species called *Streptomyces peucetius* and is under the anthracyclines antibiotic agents [91]. Chemically, DOX comprises a naphthacenequinone nucleus joined via glycosidic linkage to an amine sugar called daunosamine [88]. The molecular mechanisms of DOX are not well understood. The proposed anti-neoplastic mechanisms of DOX are by DNA intercalation, which interrupts topoisomerase-mediated repairs, and the generation of reactive oxygen species (ROS), which mediate cellular damages (cellular membranes, DNA, and proteins) [92]. [93] [94]. Regarding DNA intercalation, DOX can introduce torsional stress into the polynucleotide structure, destabilizing nucleosome structures and leading to nucleosome removal and replacement [95] [96]. Also, DOX DNA intercalation can disrupt the topoisomerase-II (specifically Topoisomerase-II α [Top-II α]) enzyme activity by preventing attach of topoisomerase-mediated DNA breaks, consequently inhibiting replication and transcription and inducing apoptosis [97] [98].

DOX has been shown to have a multiphasic disposition after administering this drug intravenously (IV) in cancer patients [88]. As a single agent, the most commonly used dose is 60 to 75 mg/m² administered at 21-day intervals [99]. Also, when DOX is combined with other chemotherapies (such as Cyclophosphamide

and Paclitaxel), the dose is usually reduced to 40 to 60 mg/m² [99]. Dox is extensively distributed in the plasma and the tissues. Accordingly, after DOX administration (IV), it is converted into its active metabolite, doxorubicinol, by utilizing an enzyme NADPH-dependent aldo-reductases. The half-life distribution is 5 min because of the fast tissue uptake. Nonetheless, the terminal half-life distribution is slow (20-48h) because of the slow elimination from the tissue [88] [100]. The absorption occurs in the cell and attaches to the cellular components. Regarding elimination, DOX and its metabolites are excreted from the bile unchanged and the urine after 5 days of DOX administration [88]. For example, in obese patients, the clearance of DOX and its metabolites decreases, which leads to toxicity due to enhanced vancomycin guideline recommended area under the curve (AUC) levels in obese patients.

Therapies side effects and Cardiotoxicity

Each treatment has different side effects, and each patient might react differently to these side effects. The most common side effects from cancer treatments such as chemotherapies include fatigue, nausea, vomiting, hair loss, risk of infection, and risk of allergic reaction. Furthermore, the most severe adverse effect of chemotherapeutic agents is damage to the heart vasculature, leading to heart failure (HF), structural damage, and hypertension. Also, other adverse side effects might include allergic reactions and the risk of leukemia in the future. Damage to heart tissue caused by chemotherapies, termed cardiotoxicity, prompted the establishment of the field of cardio-oncology to minimize

cardiovascular morbidity and mortality in cancer survivors. Although chemotherapy is very effective in treating cancer, it often has limited clinical use due to its toxicity, primarily to the heart [101]. Many factors contribute to the cardiotoxicity outcomes of therapies, such as type of drug, dosing regimen, route of administration, cumulative dose, combination with radiation therapy or other cardiotoxic medications, and patient factors such as age, sex, and previous exposure to radiation and pre-existing cardiovascular (CV) diseases and risk factors [102]. Cardiotoxicity can be classified into reversible and irreversible categories [103]. The type 1 category (direct damage) is characterized by irreversible cardiac damage that usually occurs from a cumulative dose (dose-dependent) [104] [88]. Furthermore, reversible damage is classified as a type 2 category not associated with the cumulative dose [104] [88]. On the other hand, clinical symptoms of cardiotoxicity include shortness of breath, chest pain, heart palpitations, fluid retention in the legs, stomach distention, and dizziness [104] [88].

It is well known that cardiovascular disease (CVD) is the leading cause of death worldwide and in the United States among women and men. Clinical trials of breast cancer patients overexpressing human epidermal growth factor receptor 2 (HER2) treated with trastuzumab and patients treated with anthracyclines show that cancer therapy-induced cardiotoxicity, specifically a reduction of left ventricle ejection fraction (LVEF) with or without symptoms or signs associated with HF such as a reduction in $LVEF \geq 5\%$ or an $LVEF \leq 55\%$ or a decrease in $LVEF \geq 10\%$ or an $LVEF \leq 55\%$ [105] [106]. [88]. Moreover, cardiac dysfunction associated with chemotherapies is also characterized by different types of arrhythmias and

abnormalities in ventricular repolarization and QT intervals, acute coronary syndromes, pericardial reaction (pericarditis) or alterations in myocardial (myocarditis) function that develops any time from the initiation of treatment up to two weeks after treatment [107] [108]. **Table 2** enlists some cardiotoxic effects induced by several chemotherapeutic agents, including anthracyclines, that impair autophagy & increase endothelin.

Anthracyclines (DOX)-Induced Cardiotoxicity

It is well known that Anthracyclines like DOX cause dose-dependent cardiac toxicity, either in acute or chronic settings, if used above the dose range of 400 mg/m² to 700 mg/m² [103] [104]. Historically, anthracycline-induced left ventricular dysfunction reduced LVEF and symptomatic heart failure [109]. Clinical studies show that the probability of developing cardiac dysfunction is from 1 - 2% at 300 mg/m² (cumulative dose) up to 6 - 20% at 500 mg/m². The same studies demonstrated that the probability of developing congestive heart failure (CHF) was 3% at 430 mg/m² and 21% at 728 mg/m² [110] [111].

There are many risk factors associated with increased susceptibility of DOX-induced cardiotoxicity [110] [112] such as 1) treatment with other cardiotoxic chemotherapies including cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) and trastuzumab [113] [114], 2) previous treatment with thoracic radiation, 3) age >65 years or <4 years [112], 4) hypertension and other cardiovascular [114] disease 5) preexisting disease [114] and 5) gender (female) [112]. For example,

patient age at the time of Dox treatment may influence the incidence of heart failure across all cumulative dose ranges [115].

Molecular Mechanism of Anthracyclines (DOX)-Induced Cardiotoxicity

Despite progress in the clinical understanding of DOX-induced cardiotoxicity, the absolute mechanism remains poorly understood and controversial. Several studies suggest multiple mechanisms that contribute to DOX-induced cardiotoxicity or heart failure, such as 1) increased oxidative stress in cardiac tissue, which leads to the generation of reactive oxygen species (ROS), 2) apoptosis, 3) fibrosis, 4) calcium homeostasis, and others **(Figure 3)** [88] [92] [116] [117] [118] [119]. A better understanding of DOX-induced cardiovascular injury may lead to improved treatments that specifically target the mechanisms of cardiotoxicity in patients.

1. **Oxidative stress / reactive oxygen species (ROS):** The burden of oxidative stress is observed clinically immediately after DOX administration. Studies suggest that cardiac injury by DOX is directly related to increased oxidative stress in cardiac tissue, leading to the generation of ROS and lipid peroxidation, which causes damage to cardiac muscle due to reduced levels of antioxidants and sulfhydryl groups [94] [120]. Several molecular mechanisms have been proposed for the generation of ROS from DOX and its damaging effects on cardiac tissue through the redox cycling of its quinone moiety with nicotinamide

adenine dinucleotide phosphate (NADPH) oxidase (NOX), nitric oxide synthase (NOS), iron (Fe), copper (Cu) to form the highly reactive semiquinone intermediate, leading to the generation of a superoxide and mitochondria-dependent production of ROS[116] [121] [122] [123] [124] [125] [126].

NOX-dependent ROS: NOX displays a multicomponent enzyme complex comprising membrane-bound cytochrome b-558 that plays a vital role in the formation of the semiquinone radical DOX from the transfer of an electron from NADPH to DOX [116, 127]. The isoforms of NOX1 and NOX2 play a crucial role in Dox-induced cardiotoxicity. Studies demonstrated that NOX2 deficient are resistant to DOX-induced cardiotoxicity with reduced production of superoxide radicals in cardiac tissue compared to wild-type mice exhibiting cardiac dysfunction (atrophy, cardiomyocytes programmed cell death, and interstitial fibrosis) [124, 128, 129].

NOS-dependent ROS: Moreover, NOS enzymes are members of a family of enzymes that catalyze the production of nitric oxide (NO) from L-arginine, which modulates many physiological functions, including vascular tone, neural development, and immune response [130]. There are three types of NOS (nitric oxide synthase) in mammal systems: endothelial NOS (eNOS) resides in the endothelium, inducible NOS (iNOS) resides in the immune and cardiovascular systems, and neuronal NOS (nNOS) resides in neurons and skeletal muscle [131,

132]. Doxorubicin binds to the eNOS reductase enzyme and induces DOX semiquinone radical formation; this radical reduces the free oxygen to superoxide (O_2^-) radical and is associated with DOX-induced apoptosis [133]. The role of iNOS in the pathogenesis of Dox-induced cardiac dysfunction is controversial. The DOX administration increases the iNOS transcription and protein expression and induces the formation of nitrotyrosine (NT) with increased mitochondrial superoxide levels in cardiac tissue [134-136]. NOS enzymes are a critical target in reducing Dox-mediated reactive nitrogen species (RNS).

Fe and Cu Dox-induced and production of ROS: The involvement of Fe and Cu in redox cycling generation of superoxides plays a critical role in DOX-induced production of ROS. The Fenton reaction happens (; DOX semiquinone radical forms a complex with the metal ion, which generates superoxide upon dissociation [125]. The interaction of Dox with Fe is complex; Doxorubicinol (DOX metabolite) removes iron from the Fe-S cluster of cytoplasmic iron regulatory protein 1 and converts this enzyme to a non-functional protein [137] [138]. Transferrin mRNA is then increased and prevents the translation of the iron-sequestering protein, causing a reduction in the iron regulatory protein gene that can increase the level of free iron, leading to the generation of ROS and, finally, to cardiotoxicity [138] [139].

Mitochondrial-dependent ROS production: The role of the mitochondria response to DOX in generating oxidative stress and apoptosis is

extensively studied [140]. Due to the positive charge, DOX mainly targets mitochondria, causing damage to the cardiomyocytes [141] [142]. DOX can engage into the inner mitochondrial membrane and form an irreversible complex with the protein cardiolipin [143] [144] [145]. Cardiolipin is essential in proper mitochondrial function by binding to electron transport chain complexes [146]. The DOX-cardiolipin complex causes drug accumulation in mitochondria, which disrupts the appropriate cardiolipin protein interface and leads to superoxide radical formation [141] [144] [145] [147]. Additionally, DOX administration can affect other proteins in the mitochondria, leading to impaired mitochondrial function [141] [148]. Oxidative stress from DOX changes metabolism, which can lead to heart failure or abnormal contractility and relaxation by reducing the oxidation of long-chain fatty acids in heart mitochondria and increasing glucose metabolism [141] [149] [150] [151]. In mice, the genetic ablation of mitochondrial topoisomerase (Top-MT) mitigated Dox-induced cardiotoxicity [152]. Also, the release of mitochondrial cytochrome C can activate the highly regulated programmed cell death process [153]. Therefore, measuring mitochondrial function is an early indicator and treatment target of cardiomyocyte cell death and cardiotoxicity [154].

2. **Apoptosis:** DOX stimulates both intrinsic (internal stress-mediated) and extrinsic (external stress and death receptor-mediated) pathways of apoptosis [142] [155]. Through apoptosis, cardiomyocytes display

morphological changes, including membrane blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation, and mRNA decay [156] [157]. DOX-induced oxidative stress activates heat shock factor 1 (HSF-1) and induces heat shock protein (HSP-25), which stabilizes the p53 protein, which generates pro-apoptotic factors that cause the death of heart muscle cells [88] [158] [159] [160]. Furthermore, DOX administration regulates the Akt pathway and induces cleaved caspase-3, which causes apoptosis [116] [161]. Apoptosis plays a key role in Dox-induced cardiovascular injury and is a potential target as a therapy to mitigate DOX cardiotoxicity [162] [163] [164].

3. **Fibrosis:** In cancer cells, DOX administration blocks mRNA transfer and inhibits the synthesis of the extracellular matrix (ECM) enzyme named matrix metalloprotease-1 (MMP-1), causing a reduction in tumor cell mobility. However, DOX activates the ECM enzymes MMP-2 and MMP-9 in the heart, which show toxicity by increasing collagen formation in cardiac tissue and leading to myocardium fibrosis [165, 166]. MMP-2 and MMP-9 enzymes can activate the transforming growth factor beta (TGF- β), causing collagen deposition [167]. Transforming growth factor-beta 1 (TGF- β 1) is a master regulator of fibrosis and multifunctional cytokine that is up-regulated in many cardiovascular disease states, including cardiotoxicity [168] [169] [170] [171]. Dox-induced ROS may contribute to increased activated TGF- β 1 and a pro-fibrotic cellular

environment [171]. Therefore, TGF- β 1 and related signaling pathways are essential emerging targets for therapy to mitigate DOX cardiotoxicity. Moreover, MMP-2 and MMP-9 enzymes can activate the canonical SMAD signaling pathway (phosphor-SMAD3), which enhances collagen deposition in the cardiac muscle cells [167]. SMAD phosphorylation causes nuclear translocation of the SMAD2/3 and SMAD4 complex, where it regulates fibrotic gene expression [172]. DOX administration also increases the circulating HSP-70 and its expression in the cardiac tissue of mice, thus causing inflammation and fibrosis [173].

On the other hand, there are some cardioprotective treatments for DOX cardiotoxicity (**Table 3**) that could reduce the mortality rate among cancer patients, but their efficacy in DOX cardiotoxicity is unclear. For example, in the clinic, dexrazoxane, ACEIs, ARBs, and beta-blockers are provided to adult patients with preexisting mild Left ventricle (LV) dysfunction or to healthy patients treated with Dox doses $> 300 \text{ mg/m}^2$ [174]. These therapies are not offered to adult patients with baseline left ventricle ejection fraction (LVEF) $\geq 50\%$ unless high-dose anthracyclines are being considered [174] for patients with cardiovascular risk factors, such as hypertension or diabetes; optimization of the management of these conditions is preferred [102].

Diet-Obesity and Breast Cancer

Another issue compounded in the risk of developing cancer and chemotherapy-related toxicities is obesity [175]. More than 2.0 billion people worldwide are considered overweight or obese [176]. In the United States, over 60% of women are obese (body mass index ≥ 30). **Figure 4.** Some molecular signaling pathways regulated by obesity that influence breast cancer risk, such as oxidative stress, dysregulation of insulin signaling, increased estrogen synthesis, and altered adipokine/cytokine production [177]. Several studies have demonstrated a strong link between obesity and a greater risk of developing TNBC, which is associated with worse overall survival in women with TNBC [178] [179] [180] [181]. For example, obesity may increase the risk of developing breast cancer in postmenopausal women (risk estimate, 1.82; 95% CI, 1.55 to 2.14 for overweight and obese combined) [181]. Moreover, some studies have reported a three-fold higher breast cancer mortality rate in obese women at diagnosis, indicating the critical role of obesity in the etiology of breast cancer [180].

Furthermore, it is estimated that approximately 40% of all cancer deaths are associated with dietary factors [182]. Diet has been specifically linked to 13 malignancies (deaths of patients with prostate, colorectal, gallbladder, pancreas, endometrium, and breast cancer) [183]. The Western diet or the standard American diet, contains high amounts of refined starches, sugar, processed red meats, saturated fats, trans fats, and low amounts of fruit, vegetables, and whole grains [184]. However, the mechanisms behind this association remain unclear.

Moreover, obesity is associated with 14% of cancer deaths in men and 20% in women, although dietary intake (i.e., red meat intake) [185]

ER stress and Unfolded Protein Response (UPR) Signaling Pathway

The endoplasmic reticulum (ER) is an essential organelle in eukaryotic cells that plays a major role in producing, handling, and transporting proteins and lipids [186]. Pathological or physiologic stresses such as increased secretory load, hypoxia, low glucose, ATP depletion, or the presence of mutated proteins that cannot correctly fold in the ER can lead to ER stress [187]. The ER is overwhelmed by the accumulation of unfolded or misfolded proteins. ER stress in eukaryotic cells stimulates and evolves a group of signal transduction pathways, collectively named the unfolded protein response (UPR) **Figure 5** - (reviewed in reference [188]).

Glucose-regulated protein 78 (GRP78), also known as BiP or HSPA5 (78 kDa), is a highly abundant endoplasmic reticulum (ER) chaperone. It is already known that under non-stressed conditions, PERK, IRE1, and ATF6 are bound to GRP78 and kept inactive, preventing UPR signaling. However, under conditions of ER stress – i.e., when unfolded or misfolded proteins accumulate, GRP78 release to correctly fold them, allowing the UPR signaling pathway to become activated [187] [188] [189] [190] [191]. Current understanding of these arms are briefly described below:

- 1. PKR-like endoplasmic reticulum kinase (PERK)** is a type I transmembrane protein that phosphorylates the eukaryotic translation initiation factor 2 α -subunit (eIF2 α) [192], preventing the formation of ribosomal pre-initiation complexes and reducing cap-dependent protein translation [193]. Downstream events include the induction of ATF4, which regulates the expression of several genes, including the pro-apoptotic DNA damage-inducible transcript 3 (DDI3; also known as CHOP or GADD153) [194]. Independent of PERK-mediated ATF4 expression, PERK activation results in an antioxidant response via nuclear factor erythroid 2 related factor 2 (NRF2) induced expression of genes containing antioxidant response elements (AREs) in their promoters [195].
- 2. Activating Transcription Factor 6 (ATF6)** is translocated to the Golgi after release from the ER, where S1P and S2P proteases cleave ATF6, releasing a cytosolic bZIP domain. The cytosolic domain of ATF6 (bZIP) then migrates to the nucleus and activates transcription of ER chaperones, lipid biosynthesis, and endoplasmic-reticulum-associated protein degradation [196] [197]. These reduce the density of unfolded proteins and increase chaperone protein.
- 3. Inositol-requiring Enzyme-1 (IRE1) Figure 6** - is mainly known for endo-nucleus activity; the activation results in the unconventional splicing of XBP1-U to form the active transcription factor XBP1-S. Downstream targets of XBP1-S include p58^{IPK} (blocks PERK activity) [198] [199] and several UPR chaperones, which also lead to cancer cell proliferation. IRE1 also

phosphorylates c-Jun N-terminal Kinase (JNK), contributing to apoptosis under prolonged UPR signaling [200]. IRE1 α can activate nuclear factor kappa B (NF κ B), which leads to apoptosis and inflammation in obesity [201]. Moreover, IRE1 is a conserved regulator of the human Nrf2 antioxidant response. There are two alternative mechanisms of Nrf2 activation: MAKK activation (p38/Nrf2) and KEAP1. ROS produced by NADPH oxidase or mitochondria will cause IRE1 protein sulfenylation that activates p38 and eventually Nrf2 [202].

The Unfolded Protein Response (UPR) in Diseases

The role of the UPR in influencing cellular life/death in response to ER stress has led to intense interest in the link between UPR signaling and human diseases that arise from pathologic conditions eliciting ER stress [203]. For example, cell death (a physiological consequence of chronic ER stress) is critical to the pathogenesis of many diseases, including metabolic disease, inflammation, neurodegenerative disorders, and others (reviewed in reference [201] [204]). Furthermore, GRP78 plays essential roles in development and cell survival [205]. Also, tissue-specific knockout of GRP78 leads to cell death in adipocytes and lung epithelial cells [206] [207]. UPR plays a role in different diseases and physiological processes, including regulation of cellular metabolism, inflammation, aging, and neuronal plasticity and cognition. **Table 4** shows some human diseases in which ER/UPR-activated stress is believed to play an important role.

The three arms of the UPR, IRE1-XBP1s, PERK-eIF2 α phosphorylation-ATF4, and ATF6, are essential for tumor cell survival and growth under hypoxic conditions. The UPR, IRE1, and PERK can activate c-JUN N-terminal kinase (JNK) and NF κ B to promote inflammation and apoptosis, contributing to obesity and pancreatic β -cell death in diabetes [201]. In addition, CHOP production in the PERK pathway exacerbates oxidative stress in diabetic states and atherosclerosis to aggravate the diseases [201].

The Role of Unfolded Protein Response in Breast Cancer

Several research studies have demonstrated that UPR signaling is upregulated in many different types of cancers, including breast cancer, and is associated with the development of therapeutic resistance [187] [189] [190] [201]. In general, UPR signaling prevents cancer cells from dying, and it helps them deal with the stress of the tumor microenvironment. Therefore, decreasing UPR signaling kills cancer, but increasing UPR signaling also kills cancer. There is a specific level that promotes/helps cancer progression [191]. UPR signaling also prevents some therapies from being effective.

Breast cancer cells have high UPR signaling that promotes proliferation, survival, and metastasis. **GRP78** was shown to be elevated in all breast cancer subtypes [ER α , human epidermal growth factor receptor-2 (*ERBB1*; HER2) amplified, and triple negative] when compared with the normal surrounding breast tissue, indicating a critical role for GRP78 in driving breast tumorigenesis [208]. For example, overexpression of GRP78 increased the migration and invasion [209]

of breast cancer cells, while GRP78 knockdown reduced it [210]. Also, GRP78 overexpression in both breast cancer cells and breast tumors is associated with chemotherapy resistance [211]. Our laboratory has shown that knockdown of GRP78, using RNAi in ER+ breast cancer cells, reduces overall cellular levels of anti-apoptotic BCL2, BCL-W, and BCL-xL and restores endocrine therapy sensitivity [208]. Also, our laboratory showed that targeting GRP78 in vivo restores therapeutic sensitivity and regulates innate immunity [212]. These studies provide a strong rationale for targeting GRP78 in breast cancer.

PERK signaling has been linked to a higher tumor grade and worse patient survival [213] [214] [215]. In human ductal carcinoma of the breast in situ, p-PERK levels increase compared to normal breast tissues [216]. They are higher in TNBC cell lines than in luminal cell lines [217], suggesting that overexpression of PERK is associated with a poor prognosis for breast cancer. In addition, PERK signaling mediates hypoxia-induced breast cancer progression through regulation of homologous tribologist 3 (TRIB3), autophagy activating kinase similar to unc-51 1 (ULK1), and associated membrane protein 3 to lysosomes (LAMP3). The knockdown of these proteins has been shown to reduce cancer cell proliferation (TRIB3 and ULK1), survival (ULK1), and migration (LAMP3) in hypoxia [218] [219] [220].

IRE1 signaling is the most evolutionary conserved branch of the UPR. In response to stress, IRE1 signaling can promote either cell survival or cell death and is widely described in all breast cancer subtypes and correlates with poor prognosis [221]. Previous studies demonstrate that in patients with TNBC, high

expression of IRE1 signaling is associated with poor prognosis [217]. Furthermore, overexpression of IRE1 signaling contributes significantly to resistance to endocrine therapy [221]. It has also been shown that targeting IRE1 signaling can reduce tumor burden [222] [223] and restore endocrine therapy sensitivity in resistant breast cancer cell lines [224].

Moreover, **ATF6 signaling** is activated through post-translational translocation and cleavage mechanisms. Experimental evidence suggests that ATF6's direct role in breast cancer is limited. However, blocking ATF6 has been shown to reduce angiogenesis and tumor volume in a xenograft model of breast cancer [225] and significantly decrease estrogen-induced growth [226]. There is no molecular mechanism for these observations, but ATF6 may indirectly regulate XBP1 or GRP78.

The role of Unfolded Protein Response (UPR) and oxidative stress in cardiac diseases

Cardiovascular disease (CVD), including cardiac remodeling, heart failure, ischemia-reperfusion injury, metabolic abnormalities, cardiomyopathy, and hypertension, leads to ER stress and activates the UPR signaling pathway to combat ER stress (adaptive UPR). However, if UPR fails to restore ER homeostasis (maladaptive UPR), it contributes to cardiovascular diseases by increasing reactive oxygen species (ROS) production, inflammation, and apoptosis. It has been demonstrated that elevated ER stress in cardiomyocytes is

observed in patients with hypertrophy or heart failure [227]. Therefore, ER stress can be either a cause or a consequence of CVD.

There is a research interest in understanding the role of UPR signaling in cardiac injury since the accumulation of misfolded proteins can also contribute to vascular and cardiac diseases. Research showed that failing human hearts show elevated levels of UPR components such as XBP1, BiP, ATF4, and CHOP [228] [229] [230]. Moreover, current studies demonstrate that GRP78 governs cardiac myocyte phenotype and function under various pathological conditions [231]. Moreover, myocardial infarction stimulates GRP78 expression in the heart [232]. Outstandingly, induction of GRP78 via forced overexpression of nuclear ATF6 confers strong cardioprotection against ischemic insult [233]. Even short-term upregulation of GRP78 by pharmacological UPR inducers protects the heart from subsequent lethal ischemia [234]. Additionally, it has been shown that cardiac ischemia/ reperfusion leads to strong upregulation of the UPR and stimulates GRP78 expression both in vivo and in vitro [235]. Overexpression of GRP78 decreases ischemia/reperfusion (I/R) injury by activating the Akt [236]. Moreover, UPR signaling has a complex role in cardiac diseases and can be protective or pro-death [237].

Oxidative stress plays a significant role in the pathogenesis of different diseases, such as heart failure [238]. The reactive oxygen species (ROS) are small molecules associated with several physiological and pathological events that play an important role in antimicrobial activity, regulation of genes, and signal transduction. However, ROS can induce oxidative damage to nucleic acids,

proteins, and lipids. ROS are generated from normal intracellular metabolism in mitochondria and peroxisomes by activated NADPH oxidases, as well as from various cytosolic enzyme systems and the endoplasmic reticulum. In addition, external agents can trigger ROS production. However, enzymatic and non-enzymatic antioxidant defense systems regulate overall ROS levels to maintain physiological homeostasis. It has been demonstrated that increased ROS and oxidative stress have been linked to many diseases, such as Alzheimer's, asthma, and heart failure [238-241]. Oxidative stress and endoplasmic reticulum (ER) stress are related. Misfolding protein increases in the endoplasmic reticulum (ER) during pathological conditions such as hypoxia, low glucose, ATP depletion, oxidative stress, etc. ROS are generated throughout protein folding in the ER during electron transfer between protein disulfide isomerase (PDI) and endoplasmic reticulum oxidoreductase 1 α (ERO1 α). Moreover, ROS are increased in the ER by associating PDI with Nox4, making ROS. ROS associated with UPR signaling can also activate an antioxidant response, such as Nrf2 (**Figure 6**). Also, it can increase ROS generation by activating ERO1 or NOX. On the other hand, the primary site of calcium storage is in the ER. Still, under stress conditions, calcium flows to the mitochondrial outer membrane through calcium release channels, such as inositol 1,4,5-trisphosphate receptors (IP3R) or ryanodine receptors (RyR). Increased calcium in mitochondria will increase ROS generation. The increased calcium load and ROS in mitochondria may cause the release of proapoptotic factors and mitochondrial dysfunction.

The Inositol-requiring Enzyme-1 (IRE1): As an adaptive mechanism, the IRE1 activates several cell signaling mechanisms that lead to the induction of autophagy (cytoprotective during stress), ROS levels reduction and apoptosis inhibition. However, IRE1 can lead to inflammation and inhibit autophagy in CVD disease. When maladaptive UPR occurs, apoptosis in cardiomyocytes results from IRE1 α tumor necrosis factor receptor-associated factor 2 (TRAF2), apoptosis signal-regulating kinase 1 (ASK1), JUN N-terminal kinase 1 (JNK1) and IRE1 AMP-activated protein kinase (AMPK) and serine/threonine-protein kinase PAK2 (PAK2) pathways.

Concluding Remarks

Triple-negative breast cancer (TNBC) is a terrible disease characterized by unique biological characteristics that significantly promote progression, proliferation, survival, and metastasis—also, TNBC is characterized by unique treatment options. TNBC is ER-negative, PR-negative, and HER2 normal. Around 10-20% of all breast cancer cases are triple negative, and these types of breast cancer predominately affect young and minority women. Currently, there are limited targeted therapeutic options, limiting TNBC patients to more toxic chemotherapy regimens. These breast cancer patients are expected to develop chronic diseases, mainly in the cardiovascular system and cognitive issues, making the unique long-term health concerns of this growing population a public health priority. Breast cancer survivors face a more significant burden of non-cancer chronic diseases, such as cardiovascular disease, compared to their

cancer-free peers, leading to excess morbidity and mortality, reduced quality of life, and increased medical expenditures. Consequently, finding new strategies to prevent the harmful side effects of cytotoxic chemotherapies is essential, considering cardiovascular disease is the leading cause of death worldwide and in the United States.

On the other hand, obesity is associated with worse overall survival in women with triple-negative breast cancer (TNBC). In the United States, obesity is an epidemic that is strongly correlated with breast cancer risk, prognosis, and chemotherapy-related toxicities. Studies report a three-fold higher breast cancer mortality rate in obese women, indicating the critical role of obesity in breast cancer etiology.

The role of UPR in the cardio-oncology field has not been extensively investigated. Consequently, there is new research interest in understanding the role of UPR signaling in cardio-oncology. To find new strategies that decrease the off-target effects of chemotherapy without compromising oncologic efficacy (like cardiotoxicity). We investigated whether blockade of inositol-requiring enzyme-1 (IRE1) signaling enhances chemotherapy-mediated tumor killing while preventing chemotherapy-related cardiac toxicity in obese and non-obese preclinical breast cancer models. IRE1 is an evolutionarily conserved branch of the unfolded protein response that plays a critical role in tumor survival and may contribute to cardiac injury. Our study highlights the importance of targeting the unfolded protein response pathway (IRE1) to prevent cardiac dysfunction associated with

chemotherapy treatment in obese and non-obese patients with Triple Negative Breast Cancer.

Figures and Tables

Figure 1.

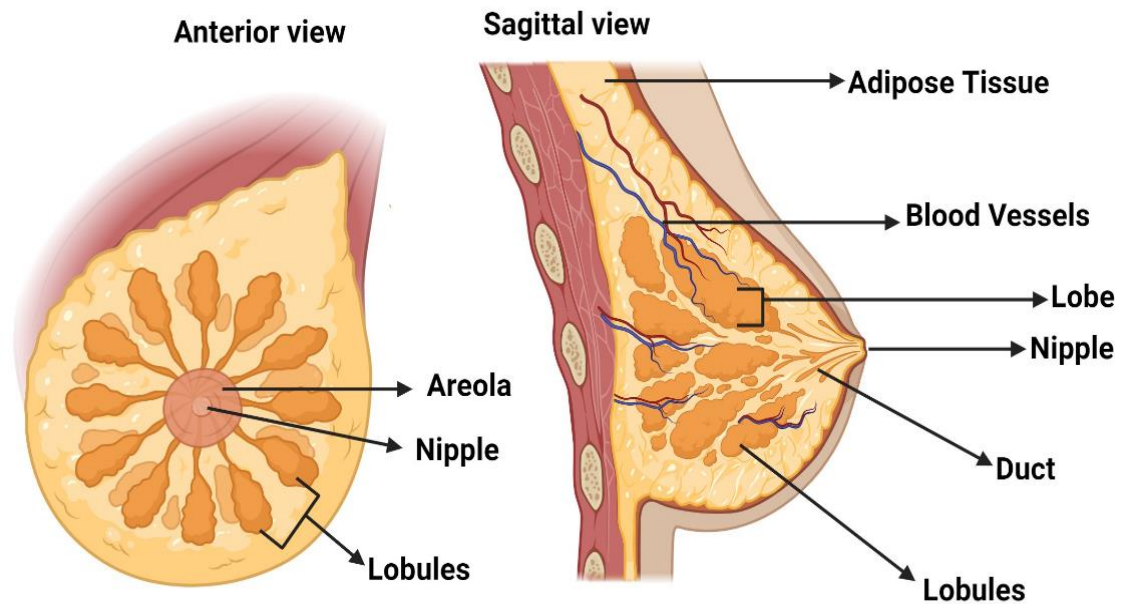


Figure 1: Female Breast Anatomy. Lobules are the glands that make breast milk. Ducts are small canals that come from the lobules and carry the milk to the nipple. The nipple is the opening in the skin of the breast where the ducts come together and turn into larger ducts so the milk can leave the breast. Areola slightly darker thicker skin that surrounded the nipple. The figure was created by www.BioRender.com, adapted from [13] and the American Cancer Society.

Figure 2.

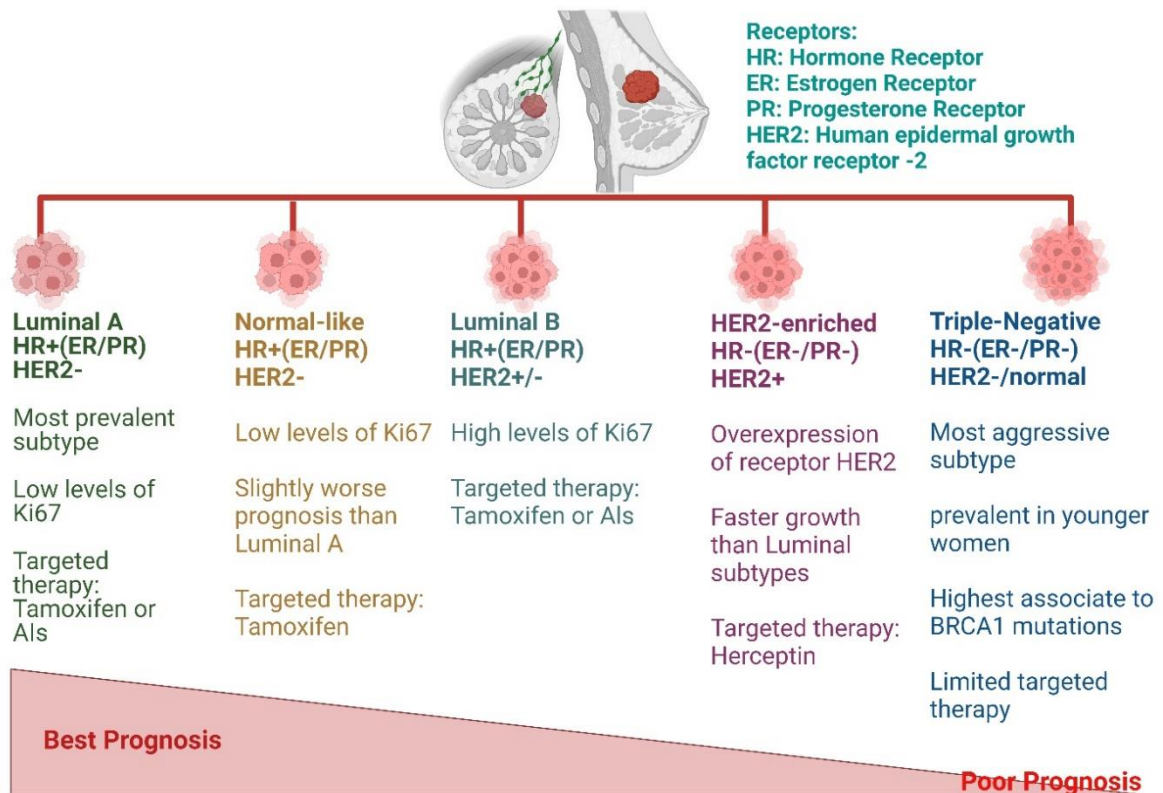


Figure 2. Molecular Subtypes of Female Breast Cancer. Advances in molecular profiling of breast tumors have identified 5 sub-categories of breast cancer based on receptor expression. Luminal A/B breast cancers account for 60-70% of all breast cancer cases and are sensitive to hormonal therapies such as Tamoxifen or aromatase inhibitors (Als). Normal breast-like account for 5-10% of all breast cancer cases. Human epidermal growth factor receptor (HER)-2 over-expressing breast cancer, accounting for 15-25% of all breast cancer cases, are sensitive to HER2 targeting therapies such as Herceptin. Lastly, basal or triple-negative breast

cancer accounts for 10-20% of breast cancer cases and is limited to toxic therapy.

The figure was created with www.BioRender.com, adapted from [29] [33] [242] [243].

Figure 3.

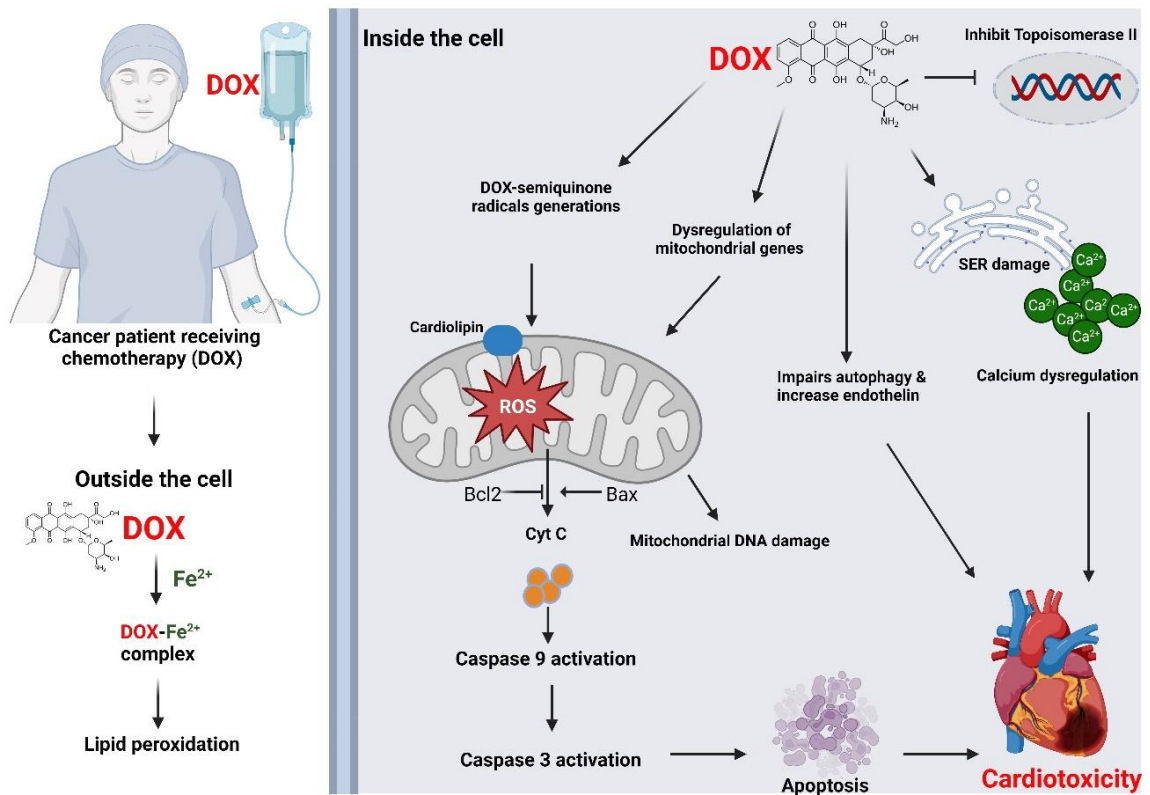


Figure 3: Different mechanisms of action of Dox-induced cardiotoxicity inside the cell and outside the cell. The figure was created with www.BioRender.com, adapted from [88].

Figure 4.

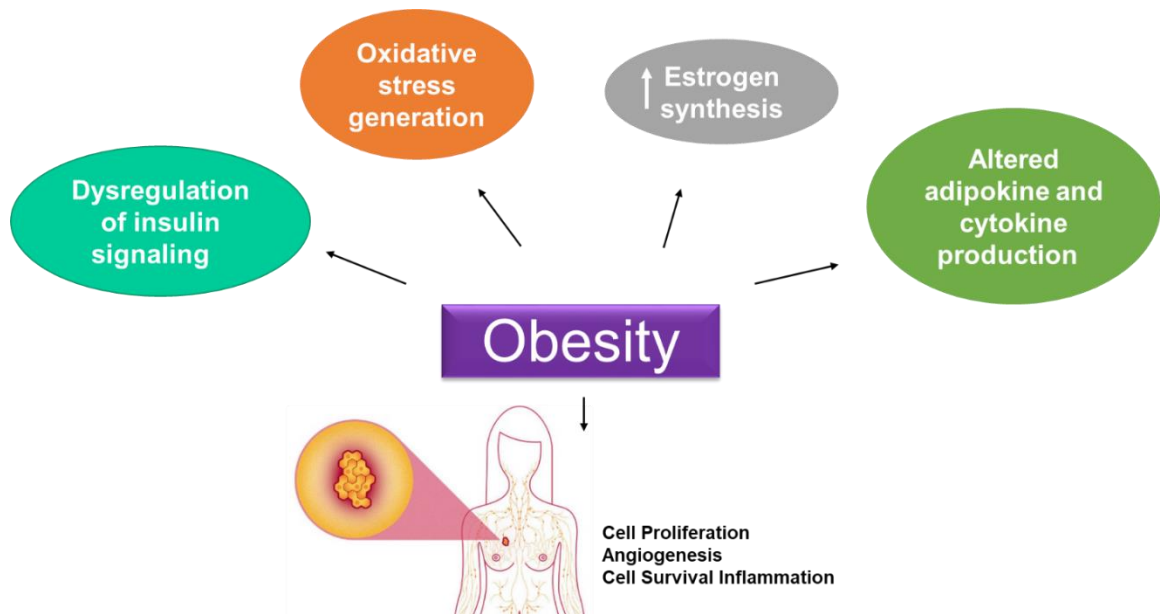


Figure 4: Molecular signaling regulated by obesity that will impact BC risk.

1) Dysregulation of insulin signaling that will lead to angiogenesis and cancer cell proliferation 2) Oxidative stress generation that regulates angiogenesis. Moreover, obesity increases 3) estrogen synthesis that leads to angiogenesis and cancer cell proliferation and 4) altered adipokine and cytokine production, leading to cancer cell proliferation, survival, and inflammation.

Figure 5.

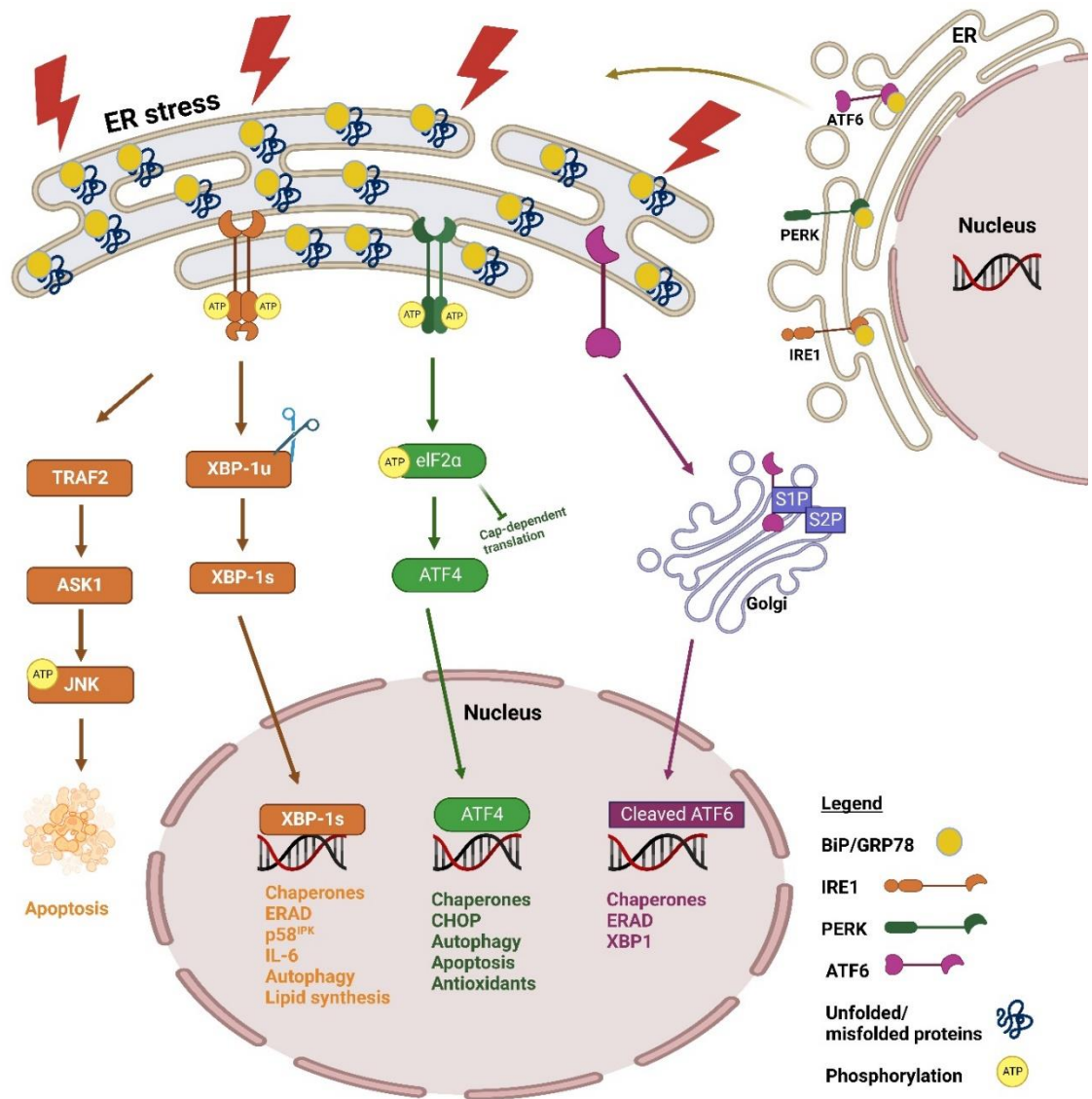


Figure 5: ER stress-induced UPR signaling activation. Summary of the three arms of the unfolded protein response (UPR) signaling pathways: inositol-requiring enzyme-1 (IRE1; ERN1), PKR-like endoplasmic reticulum kinase (PERK; EIF2AK3), activating transcription factor 6 (ATF6) are generally bound to glucose-regulated protein 78 (GRP78; HSPA5). Under ER stress, GRP78 binds unfolded proteins, releasing IRE1, PERK, and ATF6.

Figure 6.

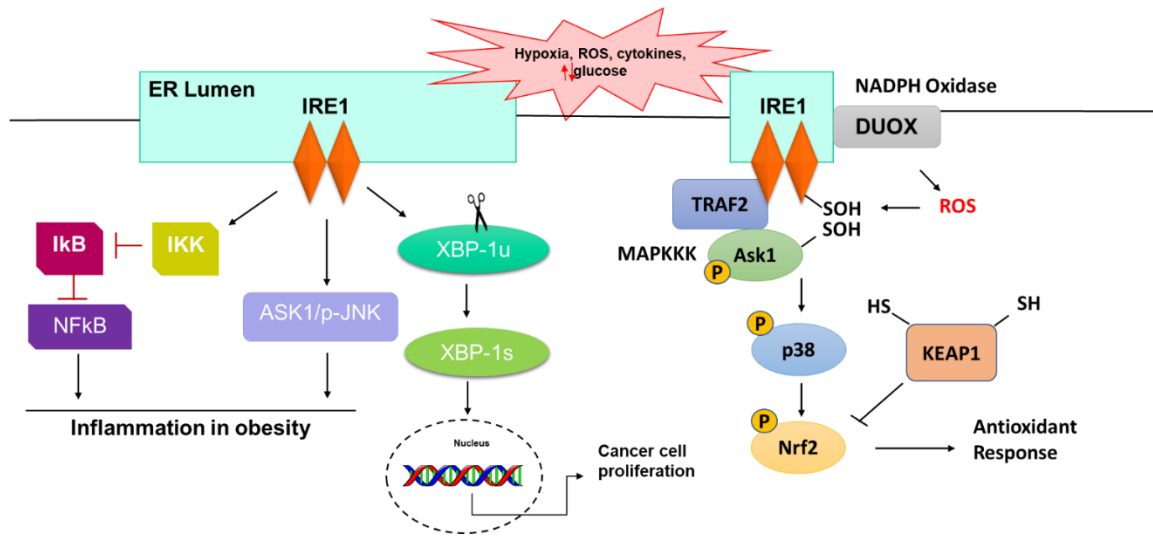


Figure 6: IRE1 Signaling Pathway: Inositol-requiring enzyme-1 (IRE1) is an arm of the unfolded protein response (UPR) pathway that plays a crucial role in tumor development. IRE1 is mainly known for endo-nucleus activities; the activation results in the unconventional splicing of XBP1-U to form the active transcription factor XBP1-S that leads to cancer cell proliferation. IRE1 can also activate MAK kinase (ASK1-pJNK) and NFκB to promote inflammation and apoptosis, which contribute to inflammation in obesity. Also, IRE1 is a conserved regulator of the human Nrf2 antioxidant response. There are two mechanisms of Nrf2 activation: MAKK activation (p38/Nrf2) and KEAP1. ROS produced by NADPH oxidase, or mitochondria, will cause IRE1 protein sulfenylation that activates p38 and eventually Nrf2, which results in an antioxidant response.

Figure 7.

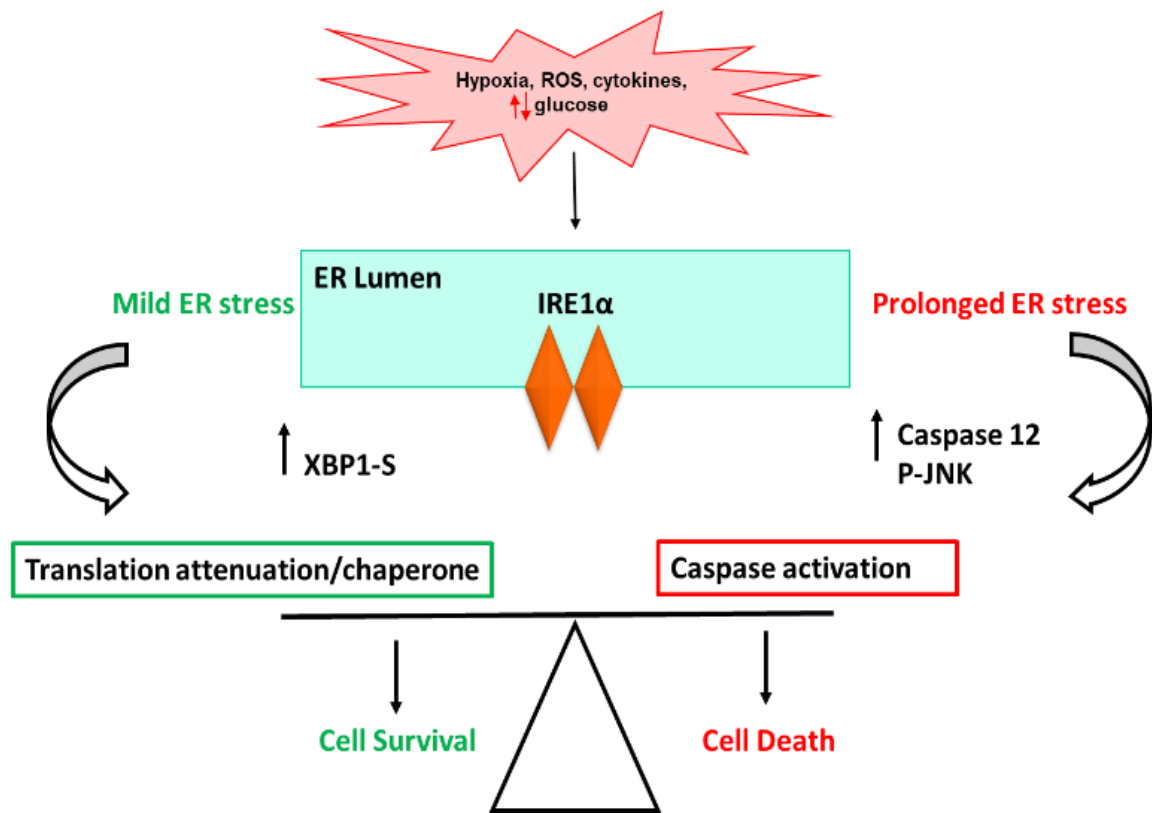


Figure 7: Role of IRE1 signaling in cardiovascular disease. There is a research interest in understanding the role of unfolded protein response (UPR) signaling in cardiac injury since the accumulation of misfolded proteins can also contribute to vascular and cardiac diseases. Moreover, the contradictory role of IRE1 signaling could be protective or pro-death. In mild ER stress, IRE1 activation results in cardiac cell survival, but in prolonged ER stress, IRE1 activation may lead to cardiac cell death through caspase activation.

Table 1

Table 1. Common types of treatments used for early-stage and locally advanced breast cancer	
Surgery	
Radiation therapy	
Chemotherapy:	
Doxorubicin	DNA intercalation and free radical damage
Epirubicin	Inhibits nucleic acid (DNA and RNA) and protein synthesis (DNA intercalation).
Paclitaxel	Blocks cell growth by microtubule inhibition
Docetaxel	It blocks cell growth by inhibiting microtubules. Docetaxel is two times more potent than paclitaxel as an inhibitor of microtubule depolymerization.
Capecitabine	Fluoropyrimidine carbamate with antineoplastic activity.
Carboplatin	Forms reactive platinum complexes that cause the intra- and inter-strand cross-linkage of DNA molecules within the cell.
Cyclophosphamide	An alkylating agent is attached to the DNA and prevents replication.
Fluorouracil (5-FU)	Inhibition of thymidylate synthase (TS) and incorporation of its metabolites into RNA and DNA

Methotrexate	It inhibits dihydrofolate reductase, preventing the reduction of BH ₂ to BH ₄ . This leads to nitric oxide synthase uncoupling and increased sensitivity of T cells to apoptosis.
Combinations of drugs used as neoadjuvant or adjuvant therapy:	
ACT (doxorubicin, cyclophosphamide, docetaxel)	
TC (docetaxel and cyclophosphamide)	
Capecitabine (Xeloda)	
EC (epirubicin, cyclophosphamide)	
AC (doxorubicin and cyclophosphamide)	
AC or EC followed by T (paclitaxel or docetaxel) or the reverse.	
CAF (cyclophosphamide, doxorubicin, and 5-FU)	
CEF (cyclophosphamide, epirubicin, and 5-FU)	
CMF (cyclophosphamide, methotrexate, and 5-FU)	
AC or EC followed by T (paclitaxel or docetaxel) and carboplatin, or the reverse	
Hormonal therapy:	
Tamoxifen	It is an estrogen antagonist, a drug that blocks the effects of estrogen, and is given most often to premenopausal women with hormone-positive breast cancer.
Aromatase inhibitors (AIs)	It is a drug that lowers the estrogen level in

	the body by blocking the aromatase enzyme. It is often given to postmenopausal women with hormone-positive disease.
Ovarian suppression or ablation	Ovarian suppression is the use of drugs to stop the ovaries from producing estrogen. Ovarian ablation is the use of surgery to remove the ovaries.
Targeted therapy:	
Trastuzumab	Inhibits HER2 homodimerization, thereby preventing HER2-mediated signaling
Pertuzumab (Perjeta)	Inhibits HER2:HER3 dimer formation, downstream signaling, and suppressing HER2-mediated cell proliferation.
Pertuzumab, trastuzumab, and hyaluronidase–zzxf (Phesgo)	Combination drug approved for people with early-stage HER2-positive breast cancer.
Neratinib (Nerlynx)	Irreversibly binding to the intracellular domain of all active HER receptors, HER1, HER2, and HER4
Ado-trastuzumab emtansine or T-DM1 (Kadcyla)	Approved for patients with early-stage breast cancer who have had treatment with trastuzumab and chemotherapy

Olaparib (Lynparza)	PARP inhibitor, which destroys cancer cells by preventing them from fixing damage to the cells
Immunotherapy:	
Pembrolizumab (Keytruda)	Immunotherapy is approved to treat high-risk, early-stage, triple-negative breast cancer in combination with chemotherapy before surgery

Table 2

Table 2. Some of the drugs are known to cause cardiotoxicity. Modified from [88] [102]	
Drugs	Cardiovascular Damage
Anthracyclines (DOX, Epirubicin)	Arrhythmia, Myocarditis, Left ventricle dysfunction, Heart failure
Cyclophosphamide	Mitral Regurgitation, Inflammation of cardiac muscle
Taxanes (Paclitaxel, Docetaxel)	Ischemia, Hypotension, Heart failure
Cisplatin	Hypertension
5-fluorouracil, Capecitabine	Dilated cardiomyopathy, Ischemia, Myocarditis, Heart failure
Sunitinib, Sorafenib	Heart failure, Acute coronary syndrome, Arrhythmia Hypertension
Trastuzumab	Arrhythmia, Angioedema, Left ventricle Dysfunction, Heart failure
SERMs (tamoxifen, Evista, Fareston)	Thromboembolism
Bevacizumab (Avastin)	Hypertension, Congestive heart failure

Table 3

Table 3. Some cardio-protective therapies for Doxorubicin treatment.	
Review in [88].	
Therapeutic strategies	Description
Dexrazoxane (Zinecard®)	FDA-approved agents are administered as cardioprotectants in conjunction with DOX and to mitigate DOX extravasation [244]. Dexrazoxane is converted intracellularly to a ring-opened chelating agent that interferes with iron-mediated oxidative stress generation [245]. Unfortunately, other data suggest that dexrazoxane occupies Top-II α in the DOX site, thereby inhibiting the effect of DOX on the tumor [246].
Angiotensin II Blockade	The major renin-angiotensin system (RAS) peptides play a central role in the control of blood pressure homeostasis and cardiovascular and renal function, making them a target for treatments of CVD and HF [247]. Angiotensin II blockade is not FDA-approved for DOX cardiotoxicity but is a discretionary option for clinicians who forgo dexrazoxane treatment [248] [249].

Beta Blockers	The β-adrenergic receptors of the myocardium play a role in regulating heart function, and their blockade is also utilized in the palliative treatment of HF [250] [251]. β-blockers, carvedilol was proposed to attenuate DOX-mediated cardiac injury by mitigating oxidative stress, preventing mitochondrial dysfunction, and maintaining calcium homeostasis [252]. However, clinical trials show that β-blockers, carvedilol provided no benefit in LVEF decline.
Statins	Statins are commonly used in cardiovascular disorders, including atherosclerosis and coronary heart disease [253], which help lower lipid levels.
Metformin	Metformin activates the AMPK signaling pathway to reduce Dox cardiotoxicity. Metformin is used to treat type 2 diabetes mellitus [254]. However, Metformin shows cardioprotective effects in rat cardiomyocytes in DOX-treated rats.
Resveratrol	Resveratrol is a powerful antioxidant classified as a polyphenol most known for its presence in grape skins. Resveratrol has potential chemopreventive activities and cardioprotective activity through the activation of AMPK [255]. AMPK activation

	attenuates oxidative stress and apoptosis and ameliorates fibrosis in Dox-induced cardiotoxicity.
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Table 4

Table 4. Role of UPR signaling pathway in diseases - reviewed in reference [201] [204]		
Diseases	ER stress/UPR branch	References
Amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), and Huntington's disease (HD)	IRE1 α /XBP1, p-eIF2 α /PERK	[256] [257] [258] [259] [260] [261] [262] [263] [264] [265] [266]
Alzheimer's disease (AD)	IRE1/XBP1/JNK2, p-eIF2 α /PERK	[267] [268] [269]
Brain Ischemia and Epilepsy	ER stress, eIF2 α /PERK, IRE1 α	[270] [271] [272]
Neuropsychiatric Diseases	ER stress	[273] [274] [275]
Eye Diseases	ATF6, GRP78, IRE1 α /XBP1	[276] [277] [278]
Skin Diseases	ER stress, XBP1	[279] [280] [281]
Metabolic and Inflammatory Diseases	IRE1 α /XBP1/JNK	[201] [282] [283] [284]
Diabetes and β -Cells	ER stress, IRE1 α /XBP1/JNK, PERK,	[285] [286] [287] [288] [289]

Atherosclerosis and Heart Diseases	ER stress, GRP78, IRE1α/XBP1, PERK, ATF6	[290] [291] [292] [293] [294] [295] [296] [297] [231] [232] [233] [234] [236] [298]
Lung Diseases	ER stress, PERK/CHOP	[299] [300] [301] [302]
Kidney Disorders	ER stress	[303] [304] [231]
Cancer	ER stress, GRP78, IRE1α/XBP1/JNK, PERK/CHOP, ATF6	[187] [189] [190] [201] [208] [209] [217] [305] [306] [307] [308]

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CHAPTER II

Targeting inositol-requiring enzyme-1 (IRE1) signaling impacts triple-negative breast cancer chemotherapy sensitivity and prevents cardiac toxicity from chemotherapy

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Targeting inositol-requiring enzyme-1 (IRE1) signaling impacts triple-negative breast cancer chemotherapy sensitivity and prevents cardiac toxicity from chemotherapy

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Abstract

Background: Triple-negative breast cancer (TNBC) is a highly aggressive breast cancer subtype that predominantly affects young and minority women. TNBC patients are more likely to receive cytotoxic chemotherapy regimens. This can result in severe side effects, such as chronic cardiac dysfunction. Another issue compounded in the risk of developing cancer and chemotherapy-related toxicities is obesity. Obesity is associated with worse overall survival in women with TNBC. Inositol-requiring enzyme-1 (IRE1) is an arm of the unfolded protein response (UPR) pathway that plays a crucial role in tumor development and progression.

Objective: Determine whether targeting IRE1 can prevent the onset of cardiac dysfunction in TNBC models undergoing chemotherapeutic treatments in the context of obesity.

Methods: Female 8-week-old BALB/c mice were placed on control (low-fat) and Western (high-fat) diets for 4 weeks. Then, mice were injected with 1×10^6 4T1-luciferase TNBC cells into the mammary fat pad. Once tumors reached 100 mm^3 , mice were treated with 1 x weekly 50 μM IRE1 mouse anti-sense morpholino (targets IRE1) for 3 subsequent weeks (I.P.) Mice were also treated with 1 x weekly 3.3 mg/kg doxorubicin (I.V.) for 3 weeks. Cardiac function was measured by ultrasound at baseline and at the end of the study. At the end of the study, tumors, hearts, and lungs were removed and weighed.

Results: Our data demonstrate that targeting IRE1 in Western diet-fed mice reduced tumor growth, potentiated doxorubicin anti-cancer activity, and increased

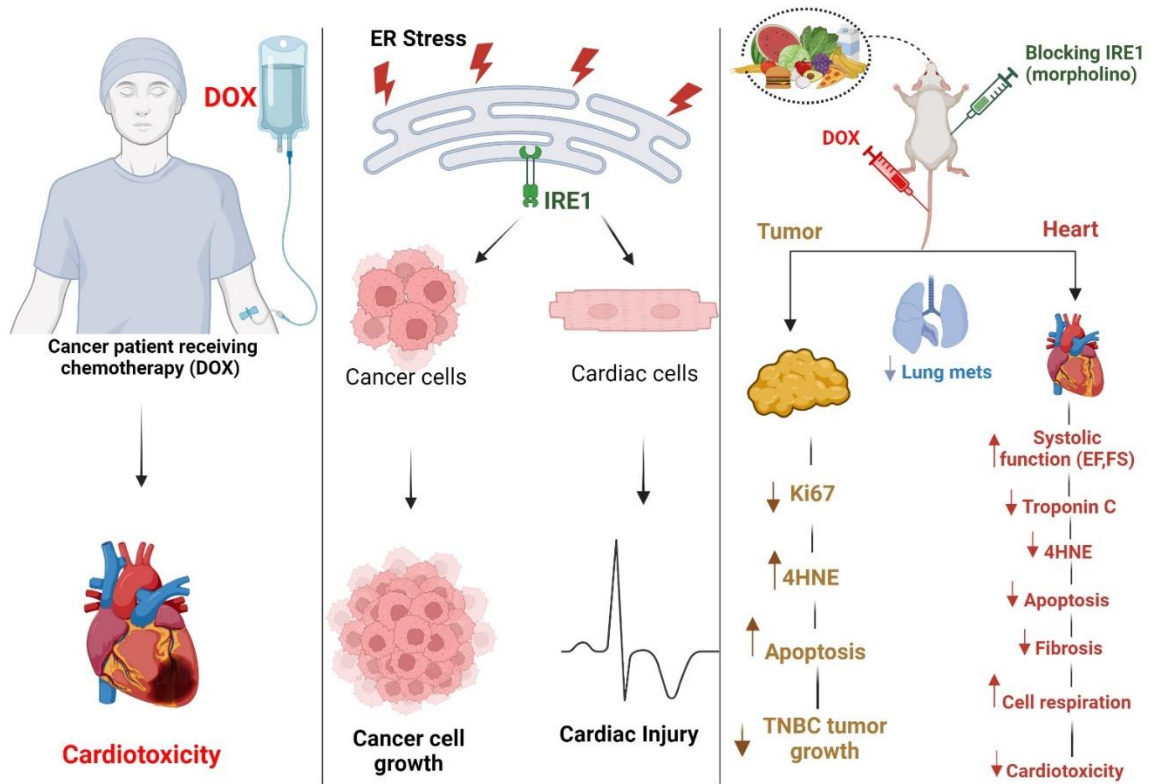
tumoral oxidative stress and apoptosis. IRE1 blockade reduced lung metastasis formation in Western diet-fed mice. Furthermore, targeting IRE1 prevented chemotherapy-mediated cardiotoxicity in control and Western diet-fed animals. The amelioration of chemotherapy-induced systolic dysfunction mediated by IRE1 targeting was associated with reduced cardiac fibrosis and decreased cardiac cleaved caspase-3. IRE1-targeting in H9C2 rat cardiac cells protected cells from doxorubicin cytotoxicity and shifted respiratory capacity, suggesting IRE1-targeting may result in the protection of mitochondria in cardiac tissue.

Conclusions: Systemic suppression of IRE1 protected cardiac tissue in mice treated with doxorubicin while enhancing anthracycline-mediated tumor killing.

Condensed Abstract (100 words-Clinical Implications)

Triple-negative breast cancer (TNBC) patients are more likely to receive anthracycline chemotherapy regimens, which are associated with the development of cardiac dysfunction. Due to the clinical benefit of chemotherapy, it is essential to find novel strategies to ameliorate toxic side effects while not limiting anti-tumor efficacy. Our data indicate that targeting the inositol-requiring enzyme-1 (IRE1) signaling pathway potentiates anti-cancer chemotherapy activity while concurrently protecting the heart from damage, thereby identifying better treatment options for TNBC patients to improve overall survival and quality of life.

Graphical Abstract



Abbreviation List

AC-T: Anthracyclines plus Cyclophosphamide and Taxanes

ASK1: Apoptosis signal-regulating kinase 1

BC: Breast cancer

CVD: Cardiovascular disease

DAB: 3,3'-Diaminobenzidine

DMEM: Dulbecco's Modified Eagle Medium

DOX: Doxorubicin, an anthracycline-based chemotherapy

ER: Endoplasmic reticulum

ERAD: Endoplasmic reticulum-associated protein degradation

FBS: Fetal bovine serum

H&E: Hematoxylin and eosin

Hmox1: Heme oxygenase-1

I.P.: Intraperitoneal

I.V.: Intravenous

IL-1 β : Interleukin-1 β

IRE1: Inositol-requiring enzyme-1

IRE1M: Inositol-requiring enzyme-1 morpholino, inhibits IRE1 translation

KEAP1: Kelch-like ECH-associated protein 1

MAPK: Mitogen-activated protein kinase

MUFA: Monounsaturated fatty acid

NF-kB: Nuclear factor kappa B

NLRP3: Nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3

Nqo1: NADPH quinone oxidase-1

Nrf2: Nuclear factor erythroid 2-related factor 2

OCR: Oxygen consumption rate p-JNK: c-Jun N-terminal kinase, phosphorylated

PUFA: Polyunsaturated fatty acid

RPMI: Roswell Park Memorial Institute media

SFA: Saturated fatty acid

Sxn1 Sulfiredoxin-1

TNBC: Triple-negative breast cancer

UPR: Unfolded protein response

XPB1-S: X-box binding protein 1 spliced

XPB1-U: X-box binding protein 1 unspliced

Introduction

Breast cancer is a multifaceted disease with various clinical and pathological features that significantly promote cancer progression, proliferation, survival, and metastasis. (1). According to the American Cancer Society, 310,720 new breast cancer cases are predicted to occur in 2024, with more than 42,000 predictable deaths, making breast cancer the second-highest killer of all cancer types in women in the United States (2). Triple-negative breast cancer (TNBC) is one of the most highly aggressive subtypes of breast cancer types, represents around 10-20% of all breast cancer cases, and predominantly affects young and minority women (3,4) TNBC patients are more likely to receive cytotoxic chemotherapy regimens associated with cardiac dysfunction. High cumulative doses of anthracyclines increase the risk of congestive heart failure by up to 26% (5). Anthracyclines plus cyclophosphamide and taxanes (AC-T) therapeutic regimens are also linked with the development of cardiovascular toxicities, including myocardial ischemia and bradycardia (5). It is well established that these chemotherapies cause cardiac damage. However, due to the clinical benefits of these chemotherapies, it is essential to find novel strategies to ameliorate the toxic side effects.

Obesity is a chronic metabolic disease that represents a significant worldwide concern (6,7). Cardiovascular disease (CVD) is the leading cause of death worldwide; obesity and poor diet are a major risk factor for CVD (8,9). Obesity is also associated with risk in thirteen different types of cancers, including breast cancer. Several studies have demonstrated a strong link between obesity

and increased risk of development of breast cancer, and breast cancer patients with obesity often display poor survival outcomes, especially TNBC (10-15). Previous work demonstrated that a high-fat diet promotes primary tumor growth and affects the development of doxorubicin (DOX)-induced cardiac dysfunction by increasing fibrosis. However, the specific mechanism remains unclear (12). The metabolic signaling associated with obesity can activate cellular stress pathways, including the endoplasmic reticulum unfolded protein response pathway (UPR) (16). In particular, inositol-requiring enzyme-1 (IRE1), an arm of the UPR, plays a crucial role in tumor development, progression, and response to therapy (17-19). IRE1 is mainly known for endonuclease activities; the activation results in the unconventional splicing of XBP1-U to form the active transcription factor XBP1-S that can lead to cancer cell proliferation and survival (20,21). However, IRE1 can also activate MAPK kinase (ASK1-pJNK) and NF κ B to promote inflammation and apoptosis, which contribute to inflammation in obesity (22,23). On the other hand, the role of IRE1 signaling in cardiac injury is less clear. Conflicting reports suggest IRE1 signaling in cardiac tissue may be protective or pro-death (24). IRE1 is a conserved regulator of the Nrf2 antioxidant response (25). NRF2 translocates to the nucleus and, after ubiquitination by KEAP1 in the cytosol, stimulates the transcription of cardio-protective genes such as Hmox1 (Heme oxygenase-1), Nqo1 (NADPH quinone oxidase-1), Sxn1 (Sulfiredoxin-1) and Peroxiredoxin (26).

The overall goal of our study was to determine whether blockade of IRE1 signaling enhances chemotherapy-mediated tumor killing while preventing chemotherapy-related cardiac toxicity in TNBC preclinical breast cancer models in

the context of obesity. We show that combining IRE1-targeting with DOX enhanced anti-tumor chemotherapy responsiveness while protecting the heart in control and Western diet-fed TNBC preclinical models.

Material and Methods

Cell culture

4T1-luciferase-tagged (TNBC) cells were obtained from ATCC (CRL-1446). 4T1 cells were cultured in RPMI media supplemented with 10% FBS penicillin/streptomycin and glutamine kept at 37 °C and 5% CO₂. H9C2 rat cardiac myoblast cell line was obtained from ATCC (CRL-1446) and cultured in DMEM media supplemented with 10% FBS penicillin/streptomycin and glutamine kept at 37 °C and 5% CO₂.

Diet composition

Prolab IsoPro RMH 3000 diet or standard diet (control diet) contains approximately 22% of proteins, 5% of fat (ether extract), 7% fat (acid hydrolysis), and 4% of fiber. The Western diet (TD. 190099) was formulated and purchased from Envigo. Western diet contains approximately 45% kcal from fat, 18% sucrose, 7% fructose, 7% cellulose, and 3.4% NaCl. Approximate fatty acids (% total): 41.2% saturated fatty acid (SFA), 34% monounsaturated fatty acid (MUFA), and 24.8% polyunsaturated fatty acid (PUFA).

***In vivo* murine TNBC tumor model**

Female 8-week-old BALB/c mice (n=80) were purchased from Charles River (Charleston, South Carolina) and placed on a control (low-fat, n=40) or Western (high-fat, n=40) diet for 4 weeks. EchoMRI and a glucose tolerance test were performed before tumor cell injection. At 12 weeks of age, mice were injected with 4T1-luciferase breast cancer cells (1×10^6 cells) into the mammary fat pad. Once tumors reached an average of 100 mm³, mice were randomized into groups. A group received 1 x weekly 50 μ M IRE1 mouse anti-sense morpholino (IRE1M; targets host (cardiac) and tumor) intraperitoneal (I.P.) for 3 subsequent weeks. Antisense morpholino is a molecule that inhibits the translation of the targeted protein (5' CAGGACGTGGCCCTGACTCC 3'). Reduction of IRE1 protein levels in tumors and hearts was confirmed by immunohistochemistry. See **Supplemental Figure S1**. Groups of control or morpholino-treated mice received 3.3 mg/kg of doxorubicin (DOX) intravenously (I.V.) after each IRE1M injection for 3 weeks. Cardiac function was measured by VEVO LAZR ultrasound system imaging in M and B modes for systolic and diastolic cardiac outputs before cell injection and 1-week post-last DOX injection. See **Supplemental Figure S2** for representative M-mode images. Tumor growth was measured every 3 days with calipers for 21 days or until humane endpoints were reached. Tumor growth was calculated by the formula $\text{volume} = W^2 \times L/2$, where W = shortest diameter and L = longest diameter. IVIS imaging (In Vivo Imaging System®, PerkinElmer; Waltham, MA, USA) was performed weekly to monitor tumor growth and metastatic lesions in the distal

organs. See the study schematic in Figure 1A. The wet weight of the tumors, lungs, and hearts was determined at the end of the study.

The Animal Care and Use Committee of the Wake Forest School of Medicine approved the protocol, and all procedures were followed per relevant guidelines and regulations.

Immunohistochemistry

Tumors, lungs, and hearts were fixed in 4% paraformaldehyde for 24 h before embedding in paraffin and cut into 5 μ m-thick sections. Tumor sections were stained against IRE1 α antibody (Novus Cat#, NB100-2323; 1:500), Ki-67 antibody (Cell Signaling Cat#, 12202; 1:800 dilution), cleaved caspase 3 antibody (Cell Signaling Cat #9661; 1:1000 dilution) and 4HNE antibody (Abcam Cat#, ab46545; 1:400 dilution) using the Dako Envision Plus IHC staining kit and visualized using DAB chromogen to investigate IRE1-targeting efficacy, tumor proliferation, apoptosis, and oxidative stress among the groups. Embedded lung tissues were stained with hematoxylin and eosin (H&E). Lung metastatic lesions were quantified, and if lesions were detected, the lesion area was measured using ImageJ software. Cross-sectional paraffin-embedded cardiac tissue was stained for fibrosis using a Picrosirius red pan-collagen staining protocol. Also, cardiac sections were stained with IRE1 α antibody (Novus Cat#, NB100-2323; 1:500), cleaved caspase 3 antibodies (Cell Signaling Cat #9661; 1:200 dilution), and 4HNE antibody (Abcam Cat#, ab46545; 1:500 dilution) using the Dako Envision Plus IHC staining kit and visualized using DAB chromogen. Staining was visualized using

the Mantra Quantitative Pathology Image System with 20× and 40× objectives; 2-4 representative images from each tissue sample were quantified and averaged.

Viability assays

H9C2 cells were plated at a density of 10,000 per well in a 96-well plate. They were transfected using 100 nM IRE1 morpholino. Twenty-four hours after transfection and plating, cells were treated with 0.25 µg/mL DOX and incubated for 24 hr. Then, the cells were stained with Hoechst. Cell viability was measured by fluorescent microscopy (BioTek Cytation, Agilent Technologies) or Cell Imaging Multi-Mode Reader, combining fluorescence and high-cont brightfield imaging with conventional multi-mode detection.

Cell respiratory measurements of H9C2 cells

The Seahorse XF96 extracellular flux analyzer was used to measure the metabolism of the H9C2 cells. Seahorse uses the mitochondrial stress test kit to measure the oxygen consumption rate or OCR, which measures mitochondrial respiration. H9C2 cells were plated at a density of 10,000 per well in a seahorse plate. Then, H9C2 was transfected using 100 nM IRE1 morpholino. Twenty-four hours after transfection, cells were treated with 0.25 µg/mL DOX and were incubated for 24 hr. Then, the cells were treated sequentially with oligomycin, FCCP, and rotenone + antimycin A. OCR is normalized against cell counts. The measurements from these assays are expressed as OCR, and an analysis was performed using Wave software.

Enzyme-Linked Immunosorbent Assay (ELISA)

Plasma was collected from the animals at the end of the study, stored at -80°C , and analyzed. The levels of B-type natriuretic peptide or BNP (Novus Cat#, NBP2-70011) and Troponin I type 3 (Novus Cat#, NBP3-00456) were measured using ELISA following the manufacturer's protocol, and the plates were read immediately at 450 nm using a Bio-Rad Benchmark Plus microplate spectrophotometer.

Results

Targeting IRE1 enhances anthracycline-mediated tumor cytotoxicity in a murine model of TNBC.

Before TNBC cell injection, the body fat composition of BALB/c mice fed a control or Western diet was measured using EchoMRI. Western diet consumption increased body fat mass percentage by 1.2 times compared to the control diet (**Fig. 1B**), establishing elevated adiposity in the diet-induced obesity model. We also demonstrated that Western diet-fed animals displayed elevated glucose area under the curve when compared with control diet-fed animals after 4 weeks of diet administration (**Fig. 1C**). In control diet-fed animals, DOX, IRE1M, and a combination of IRE1M+DOX significantly reduced tumor volume (**Fig. 1D**). However, in Western diet-fed animals only the combination of IRE1M+DOX significantly decreased tumor volume respective to saline-treated animals on the same diet (**Fig 1E**).

Tumor weight was measured (**Fig.1F**), and targeting IRE1 significantly reduced tumor weight alone or in the context of chemotherapy in control and Western diet-fed mice. Tumor size was linked with cell proliferation as measured by Ki67 staining (**Fig. 1G-H**), a cancer cell proliferation marker. Tumors from Western diet-fed mice had the highest number of Ki67-positive cells compared to tumors from control diet-fed mice. All treatment groups reduced tumor Ki67 in Western diet-fed mice, and targeting IRE1 combined with chemotherapy reduced proliferation in the Western diet by about 5-fold (**Fig. 1G**). Treatment had no significant effect on Ki67 immunoreactivity in tumors from control diet-fed animals. These results indicate that targeting IRE1 potentiates anthracycline-mediated breast tumor therapy responsiveness and reduces tumor proliferation in obesity.

Diet and IRE1 blockade enhance tumor apoptosis and contribute to oxidative stress.

Apoptosis is a programmed cell death triggered by extracellular (extrinsic apoptosis) or intracellular (intrinsic apoptosis) signals that can be measured by induction of cleaved caspase-3 (27). As expected, DOX-induced cleaved caspase 3 ($p=0.0057$) in the control diet. Targeting IRE1 in combination with DOX significantly increased cleaved caspase 3 ($p = 0.0001$) in the control diet (**Fig 2A-B**). In western diet-fed mice, only the combination of IRE1M + DOX-induced cleaved caspase 3 ($p<0.0001$) (**Fig 2A-B**), suggesting a loss of DOX alone efficacy in Western diet-fed animals that can be overcome by IRE1-targeting. Increased oxidative stress can induce apoptosis. Tumors from mice that were treated with IRE1M alone and tumors from mice treated with IRE1M+DOX showed significantly

increased 4HNE ($p<0.05$) in Western diet-fed mice (**Fig 2C-D**). These data suggest that targeting IRE1 promoted cancer cell apoptosis by increasing oxidative stress in Western diet-fed mice.

Targeting IRE1 modulates the development of lung metastases.

At the end of the study, lung weight was recorded. Targeting IRE1 in combination with DOX decreased lung weight ($p<0.05$) in mice fed the control diet (**Fig. 3A**). In Western diet-fed mice, there is no difference in lung weights between treatments (**Fig. 3A**). The lung tissues were paraffin-embedded for lung metastatic lesions by H&E staining. In mice consuming a control diet, the combination treatment of IRE1M and DOX reduces lung metastatic lesions by around 3-fold compared to the control treatment (**Fig. 3B-C**). Targeting IRE1 alone and in combination with chemotherapy significantly reduces metastatic lesions ($p<0.005$) when compared with control treatment in Western diet-fed mice (**Fig. 3B&D**). These results suggested that the combination treatment of IRE1M and DOX reduced metastatic spread, which could improve the clinical outcomes of breast cancer patients.

IRE1 targeting preserves cardiac function after treatment with DOX.

The cardiac function was measured in mice using a VEVO LAZR ultrasound system in the M and B modes (representative images are shown in **Supplemental Figure S2**). In control and Western diet-fed mice, DOX reduced left ventricular systolic function, as measured by ejection fraction (EF), compared to baseline function (**Fig 4A-B**). Interrogation of % change of EF (baseline to endpoint change) demonstrated that targeting IRE1 preserved ejection fraction after DOX treatment

($p < 0.05$) in mice fed control and Western diets when compared with DOX alone treated groups (**Fig 4A-B**). Also, we measured cardiac fractional shortening (FS), a measurement of left ventricle function in control and Western diet-fed mice (**Fig. 4C-D**). Results show that DOX treatment significantly decreased FS in control animals but was not significant in Western diet-fed mice compared to baseline measurements (**Fig. 4C-D**). However, we observed significant differences between DOX alone and DOX+IRE1M groups regarding the % change in FS in Western-diet-fed animals, suggesting improved cardiac function using FS parameters (**Fig 4C-D**). We also measured Troponin I type 3 serum levels to assess myocardial cell wall injury. We found that DOX treatment significantly increased plasma Troponin I type, which was not observed in IRE1M + DOX treated groups in both control and western diet-fed animals (**Fig. 4E-F**). We also measured plasma BNP (**Supplemental Figure S3**) but did not observe changes in this parameter with DOX treatment in either diet. To determine whether the mechanism of cardio-protection inhibits apoptosis signaling, cross-sectional paraffin-embedded heart tissue was stained for cleaved caspase 3. We found that DOX significantly increased cell death marker cleaved caspase 3 in control ($p = 0.0016$) and Western ($p < 0.0001$) diet-fed mice (**Fig. 4G-H**). However, IRE1 blockade decreased cleaved caspase 3 protein levels in cardiac tissue when combined with chemotherapy in both diet, control, and Western diets (**Fig. 4G-H**). These results suggested that targeting IRE1 with morpholino could be used to prevent cardiac dysfunction in breast cancer patients receiving anthracycline chemotherapy.

IRE1 blockade prevents cardiac tissue remodeling associated with DOX treatment

Cardiac fibrosis is indicative of cardiac stress and damage. Fibrosis can accumulate in cardiac tissue, limiting contractility and function. We measured cardiac fibrosis by assessing interstitial and perivascular fibrosis using Picrosirius red staining (**Fig 5A-D**). In Western diet-fed mice, IRE1M + DOX treatment reduced cardiac interstitial fibrosis (**Fig. 5A-B**) compared to DOX alone. Cardiac perivascular fibrosis was induced by DOX in mice on either diet (**Fig. 5C-D**), and this effect was ameliorated by targeting IRE1. Thus, the blockade of IRE1 prevented DOX-induced fibrotic cardiac remodeling.

IRE1 blockade regulated oxidative stress and cell metabolism.

DOX significantly increased the 4HNE oxidative stress marker ($p < 0.0001$) compared to the control-treated animals on either diet (**Fig. 6A-C**). IRE1 blockade significantly reduced oxidative stress in heart tissue with or without combination with chemotherapy ($p < 0.0001$) in control diet-fed animals when compared with DOX alone (**Fig. 6A-C**). Western diet-fed animals displayed elevated cardiac oxidative stress than control diet-fed animals, suggesting diet impacts baseline oxidative damage. While we did not observe a significant regulation between cardiac 4HNE in control and IRE1M+DOX-treated animals consuming the Western diet, there was a trend for reduced 4HNE. Using H9C2 rat cardiomyoblasts treated with IRE1M +/- DOX, we show knockdown of IRE1 prevents DOX-induced cytotoxicity *in vitro* (**Fig. 6D**). Since 4-HNE and oxidative stress can limit mitochondrial function, we performed cellular respiration analysis in H9C2 cells

using a Seahorse bioanalyzer. Our data show that basal respiration was reduced by approximately 50% by DOX treatment (**Fig. 6E-F**). However, treatment with DOX did not reduce basal respiration with IRE1 blockade. Furthermore, maximal respiration (calculated as basal OCR – OCR after FCCP treatment) was elevated with the DOX +IRE1 blockade when compared to DOX alone (**Fig. 6E & 6G**), suggesting that IRE1 blockade can preserve mitochondrial respiration to overcome stress as a consequence of DOX treatment.

Discussion

Our finding demonstrates that targeting the inositol-requiring enzyme-1 (IRE1) arm of the UPR pathway reduced tumor progression and prevented cardiac dysfunction associated with chemotherapy treatment in control and Western diet-fed mice with TNBC tumors, representing a novel combinatorial neoadjuvant therapy regimen to protect the heart while sensitizing the tumor concurrently. IRE1 signaling can promote cell survival or cell death in response to stress. Previous studies demonstrate that in patients with TNBC, high expression of IRE1 signaling is associated with poor prognosis (20). It has also been shown that targeting IRE1 signaling can reduce tumor burden (28, 29). In our study, we used DOX, an anthracycline chemotherapeutic agent clinically effective in treating different types of cancer, especially in patients with advanced and invasive breast cancer, such as triple-negative breast cancer. However, the limitation of DOX is that the cumulative dose can cause cardiac dysfunction that can occur either in the acute or chronic setting. Our results demonstrate that blockade of IRE1 combined with

DOX reduced the tumor volume in Western diet-fed untreated mice groups. In control diet-fed animals, there was no significant difference in tumor volumes between the different treatments, suggesting that the additive anti-tumor efficacy of targeting IRE1 and chemotherapy was enhanced in the context of obesity. Further supporting evidence indicates that while DOX and IRE1M+DOX treatments increased tumor apoptosis (cleaved caspase 3) in both diets, only reduced tumor proliferation (Ki67) was observed in tumors from mice consuming a Western diet in the different treatment groups. However, we did note a significant reduction in tumor weight at the end of the study in DOX + IRE1M treated mice on both diets, thereby demonstrating beneficial outcomes of the combinatorial treatment on either dietary background. Furthermore, we investigated intratumoral oxidative stress (4HNE) and found that it was significantly increased by targeting IRE1 alone or combined with DOX only in the Western diet. These results suggest that targeting IRE1 with morpholinos impaired IRE1-mediated signaling that may disrupt the ER chaperones, ERAD, or their functions, which affect cell survival and trigger apoptosis in TNBC cells. IRE1 has been shown to have multiple functions, including unconventional splicing of XBP1 and activation of JNK, which trigger apoptosis (30-32). In cancer cells, IRE1/XBP1 is a key regulator of ER stress that controls cell survival and/or apoptosis. Evidence suggests that inhibition of IRE1 (Rnase site) leads to decreased splicing of XBP1, which reduces cell proliferation and increases apoptosis in breast cancer (33), pancreatic cancer (34), and multiple myeloma (35). Consequently, IRE1 targeting is of interest for anticancer drug development strategies.

It has been demonstrated in the murine model of triple-negative breast cancer that 4T1 cells are injected into the mammary fat pad, causing metastasis incidence to the lungs in less than 60 days (36). We found that IRE1M, in combination with chemotherapy, reduces lung lesions in control and Western diet. However, IRE1M alone also reduced lung metastasis in the Western diet but not the control diet. These data suggest targeting IRE1M, when combined with chemotherapy, reduces tumor progression and metastatic development.

In the United States, it is estimated that there are over 3.5 million living breast cancer survivors, but they face a more significant burden of non-cancer chronic diseases, such as cardiovascular disease, compared to their cancer-free peers, leading to excess morbidity and mortality, reduced quality of life, and increased medical expenditures (5). It is well known that cumulative doses of anthracyclines cause cardiotoxicity. However, there is a gap in understanding the mechanisms of cardiotoxicity, markers of susceptibility, and complementary therapeutic strategies that ameliorate side effects without compromising oncologic efficacy. Several studies have associated cardiovascular diseases with alterations in the endoplasmic reticulum (ER), particularly disruption of ER homeostasis leading to upregulation or severe activation of the UPR, especially IRE1 (37-41). Activation of IRE1 has been shown to cause activation of the NLRP3/IL-1 β inflammatory pathway associated with inflammation in cardiovascular tissue (42). However, deletion or inhibition of inositol-requiring enzyme 1 (IRE1) activity causes a significant reduction in cardiac injury formation by reducing caspase-1 activity and IL-1 β secretion (43), and It also attenuates cardiac fibrosis by restoring

autophagy in cardiac fibroblasts (44). In conjunction with these published findings, this study investigates the effect of IRE1 blockade on cardiac function parameters, including ejection fraction and fractional shortening. We show that DOX reduced ejection fraction and fractional shortening in the control and Western diets; targeting IRE1 prevented this effect from chemotherapy in both diets. In addition, we found that IRE1 blockade reduced cardiac dysfunction marker (Troponin I type 3) and perivascular fibrosis.

The role of the mitochondria response to DOX in generating oxidative stress and apoptosis is extensively studied (45). DOX targets mitochondria, causing cardiomyocyte damage (46) by forming an irreversible complex with cardiolipin (47). Cardiolipin is an inner mitochondrial membrane phospholipid that is vital to mitochondrial function. The DOX-cardiolipin complex causes drug accumulation in mitochondria, which leads to superoxide radical formation (46-48). Hence, oxidative stress from DOX changes metabolism, which can lead to heart failure or abnormal contractility and relaxation by reducing the oxidation of long-chain fatty acids in heart mitochondria and increasing glucose metabolism (46,49-52). On the other hand, several studies showed that UPR arms have a cardio-protective effect on ischemic insults. (53-56) In our study, we showed that IRE1 blockade increased mitochondrial respiration in cardiac H9C2 cells, which could explain the cardio-protective mechanism of IRE1 blockade. Our results suggest that IRE1 blockade protects from chemotherapy-induced cardiotoxicity without reducing anti-tumor effects and, in the context of obesity, may enhance anti-cancer chemotherapy efficacy.

Study Limitations: One limitation of our study is the age of the BALB/c mice. This study models premenopausal and may not accurately represent postmenopausal women with TNBC. However, TNBC predominantly affects younger women. Another limitation is the obesity component. We used the BALB/c strain that is resistant to diet-induced obesity; while the Western diet-fed animals display an increased body fat composition and higher glucose area under the curve in response to diet, they do not technically obtain the definition of obesity in a murine model.

Clinical Perspective: This study is consistent with the general agreement in the field that DOX is a highly effective chemotherapy; however, it could be detrimental to the long-term quality of life of all cancer survivors due to severe cardiac dysfunction and the development of heart failure. For this reason, it is important to develop new strategies, such as targeting IRE1, that can provide better treatment benefits by reducing the side effects of chemotherapies, especially in younger patients receiving this toxic chemotherapy (DOX). Several studies investigate the cardiotoxicity effects of chemotherapy in non-tumor-bearing mice. We now demonstrate in a tumor model how the cardiotoxicity effect of DOX is reduced without compromising the oncological efficacy of chemotherapy by blocking IRE1.

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Figures and Legends

Figure 1.

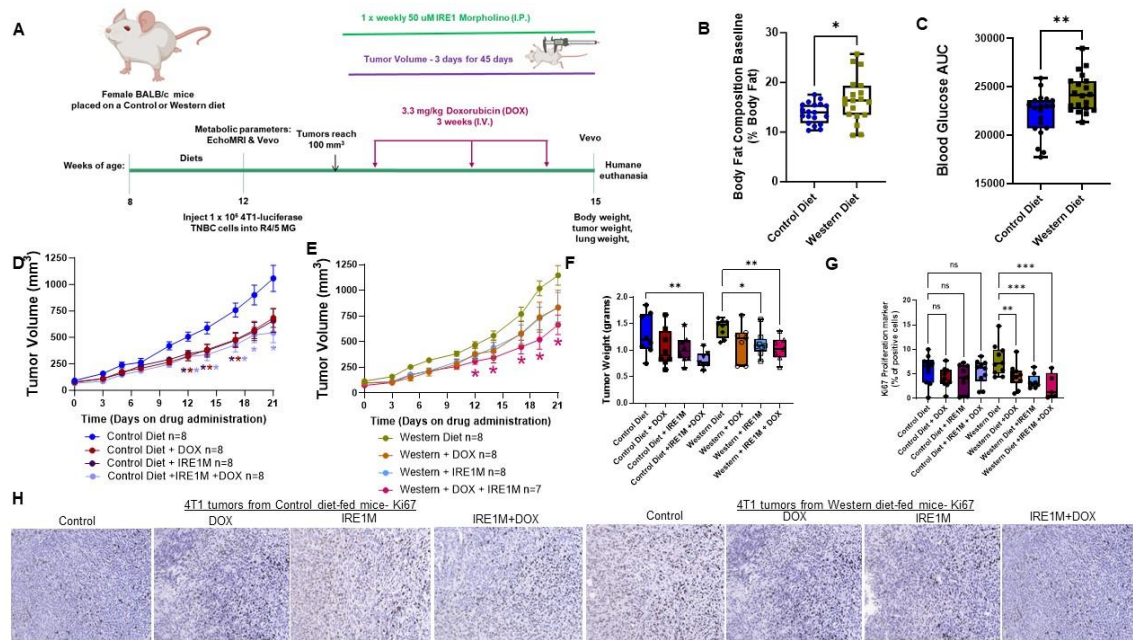


Figure 1. IRE1 blockade enhances the anti-tumor effects of DOX chemotherapy in vivo. **A.** Schedule of treatment. **B.** Body fat mass composition was determined by EchoMRI. n=19-20 per diet; *p<0.05, Unpaired t test. **C.** Glucose area under the curve n=19-20 *p<0.05 Unpaired t-test. **D-F.** BALB/c mice were injected with 4T1 cells (1 x10⁶ cells) into the mammary fat pad to induce tumor growth. Once tumors developed mice were treated with the anthracycline doxorubicin with or without anti-sense morpholino to IRE1. **D-E.** Tumor volume was measured with a caliper every 3 days and was calculated by the formula: volume=W2×L/2, where W=shortest diameter and L=longest diameter. n=7-8 per

diet; * $p < 0.05$, *denotes significant difference from control treatment of control or Western diet-fed mice; two-way ANOVA followed by a Tukey's multiple comparison test. **F.** Tumors were excised, and weights were recorded at the end of the study. $n = 7-8$; * $p < 0.05$, *denotes significant difference from control treatment from control or Western diet-fed mice; one-way ANOVA followed by a Tukey's multiple comparison test. **G.** Tumor size was correlated with cell proliferation as measured by Ki67 staining in control and Western diet which is a marker of cancer cell proliferation. **H.** Representative images of primary 4T1 tumors Ki67 in control and Western diet-fed mice. $n = 7-8$, * $p < 0.05$.

Figure 2.

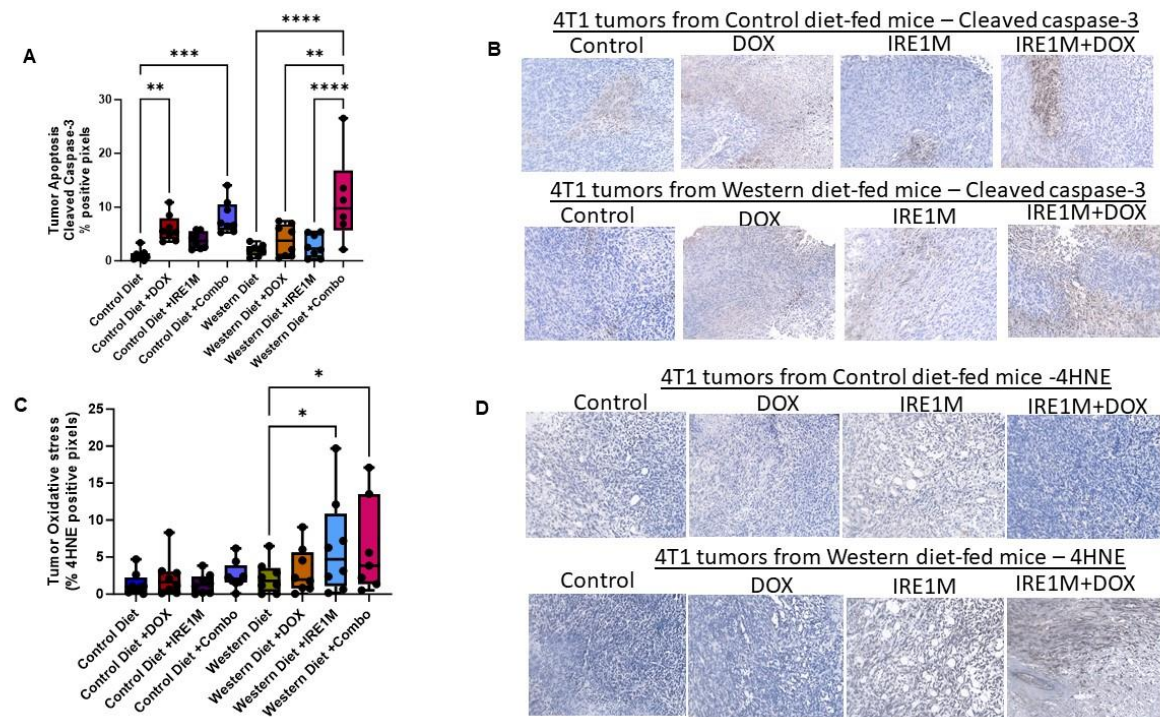


Figure 2. Targeting IRE1 stimulates cell death by increasing oxidative stress in tumor tissue. **A.** Tumor tissue was obtained from control and Western diet-fed mice, paraffin-embedded and sectioned. Sections were stained with DAB (Cleaved Caspase 3). $n = 7-8$, $*p < 0.05$; two-way ANOVA followed by a Tukey's multiple comparison test. **B.** Representative image of Cleaved Caspase 3 staining 4T1 tumors from control and Western-fed mice; $n = 7-8$, $*p < 0.05$. **C.** Tumor oxidative stress from control and Western diets-fed mice were measured by 4HNE staining. **D.** Representative image of 4HNE staining 4T1 tumors from control and Western-fed mice $n = 7-8$, $*p < 0.05$; two-way ANOVA followed by a Tukey's multiple comparison test.

Figure 3.

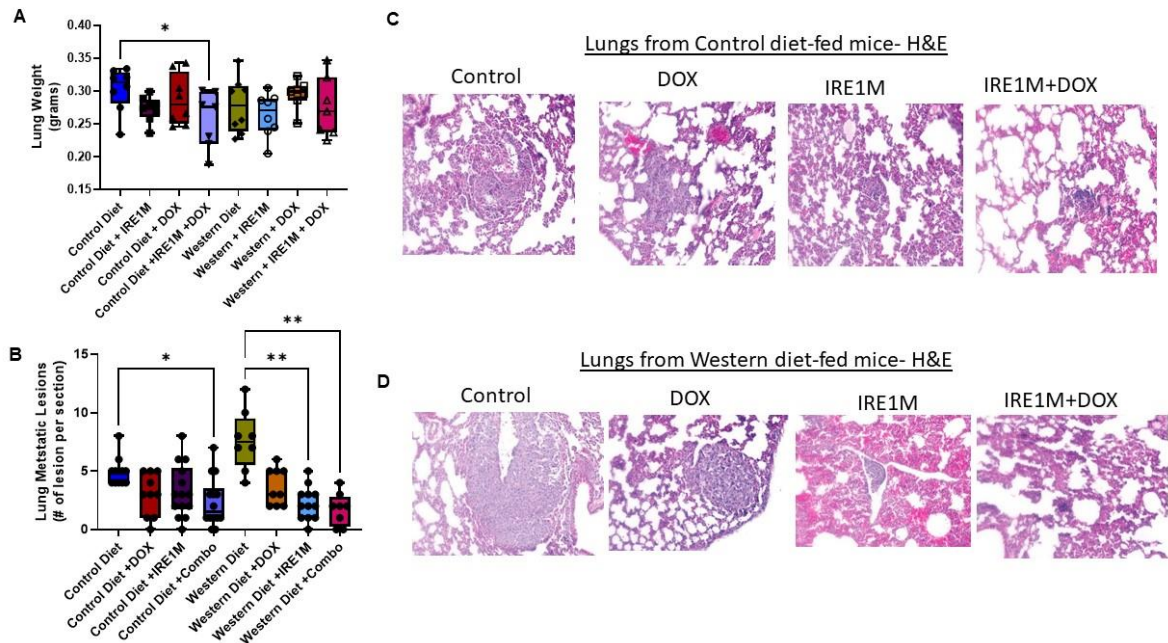


Figure 3. IRE1 blockade reduces lung metastasis. **A.** At the end of the study mice from the control and Western diet were euthanized, and lungs were excised and weighed. $n = 7-8$, $*p < 0.05$; one-way ANOVA followed by a Tukey's multiple comparison test. **B.** Lung tissue sections were paraffin-embedded and stained with Hematoxylin and Eosin (H&E) to determine tissue structure and counted as the number of lesions per section $n=9-12$; $*p < 0.05$; Brown-Forsythe and Welch ANOVA tests followed by a Tukey's multiple comparison test. **C-D.** Representative H&E images of lung tissue from control (**C**) and Western (**D**) diet-fed mice.

Figure 4.

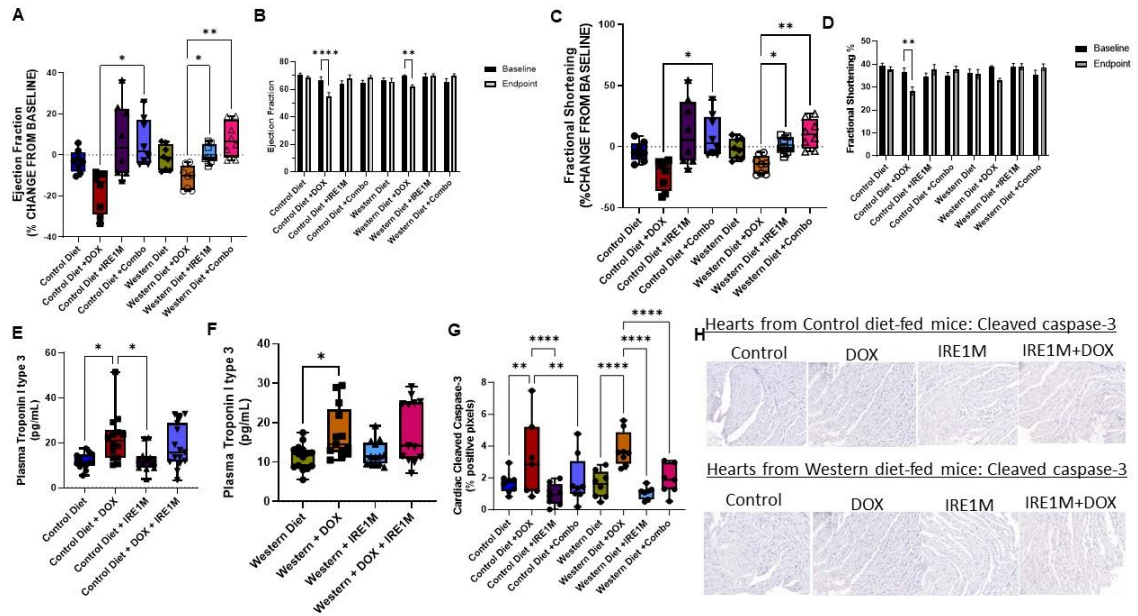


Figure 4. Targeting IRE1 prevents cardiac dysfunction from chemotherapy (DOX). M-Mode tracing with in vivo ultrasound with Vevo LAZR Photoacoustic Imaging System at baseline and endpoint. Cumulative DOX dose = 10 mg/kg. **A.** Ejection fraction in mice consuming control and Western diets at baseline, bearing primary 4T1 breast tumor, after injected I.V. with DOX (endpoint), graphed as percent change from baseline n=8. *p<0.05; Brown-Forsythe and Welch ANOVA tests followed by Tukey's multiple comparison test. **B.** Ejection fraction raw data in mice consuming control and Western diets at baseline and endpoint n=8. ****p<0.0001, **p=0.0071 two-way ANOVA followed by a Tukey's multiple comparison test. **C.** Fractional shortening in mice consuming control and Western diets at baseline, bearing primary 4T1 breast tumor, after injected I.V. with DOX

(endpoint), graphed as percent change from baseline n=8. *p<0.05; Brown-Forsythe and Welch ANOVA tests followed by Tukey's multiple comparison test.

D. Fractional shortening raw data in mice consuming control and Western diets at baseline and endpoint n=8. **p=0.0012 two-way ANOVA followed by Tukey's multiple comparison test. Plasma levels of the cardiac biomarker troponin I type 3 (cTnI) in Control **(E)** and Western **(F)** diets fed mice n=8. *p<0.05; two-way ANOVA followed by Tukey's multiple comparison test. **G.** Cardiac tissue was obtained from control and Western diet-fed mice, paraffin-embedded and sectioned. Sections were stained with DAB (Cleaved Caspase 3). n = 6-7, **p =0.0016, ****p<0.0001; two-way ANOVA followed by a Tukey's multiple comparison test. **H.** Representative image of Cleaved Caspase 3 staining hearts from control and Western-fed mice.

Figure 5.

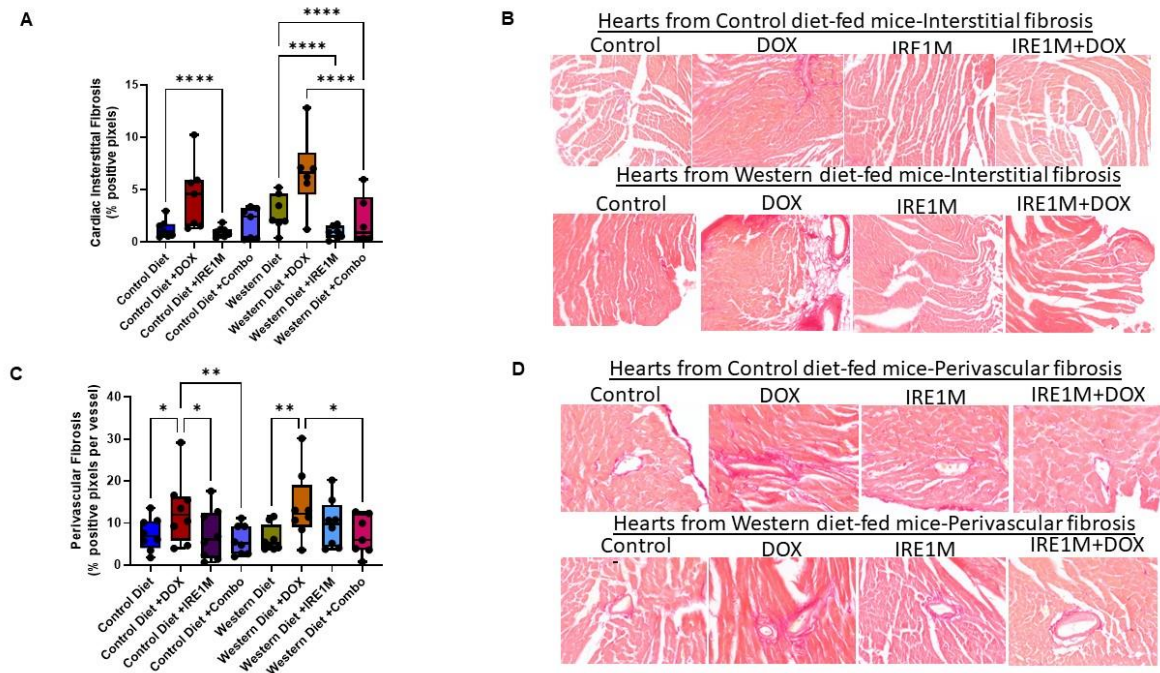


Figure 5. Targeting IRE1 modulates DOX-induced fibrosis. Hearts of mice were excised after 3 weeks of IRE1M and DOX treatment, sections were paraffin-embedded and **(A-D)** picosirius red was performed. **A.** Interstitial fibrosis in mice consuming control and Western diets n=5-7; two-way ANOVA followed by a Tukey's multiple comparison test. **B.** Representative images of picosirius red staining in cardiac tissue from mice on control and Western diets indicating collagen deposition. 40×, Scale bar = 100 μm; 4 sections/animal. **C.** Perivascular fibrosis in mice consuming diets. n=5-7; two-way ANOVA followed by a Tukey's multiple comparison test. **D.** Representative image from control and Western diet-fed mice. 40×, Scale bar = 100 μm; 4 sections/animal.

Figure 6.

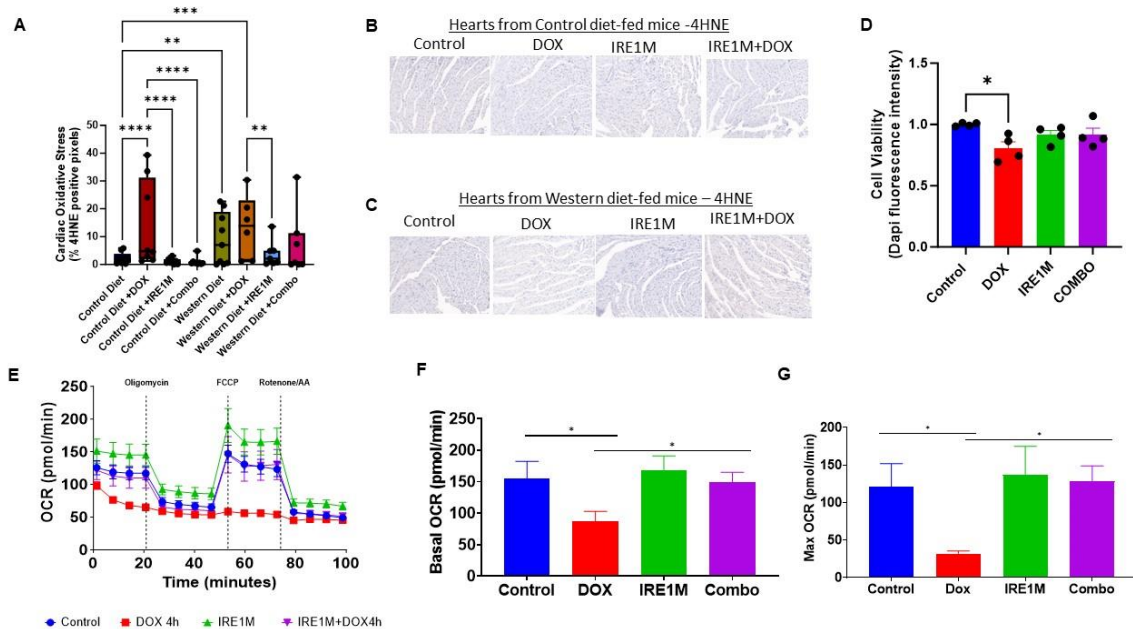
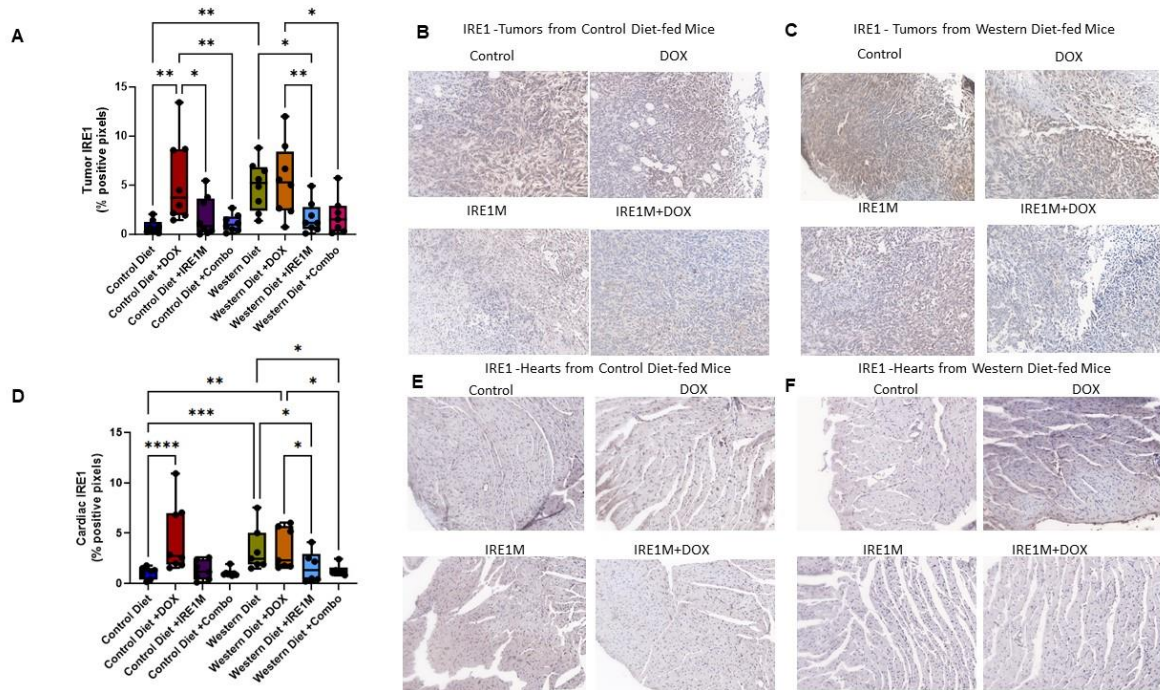


Figure 6. IRE1 blockade modulates mitochondria respiration. **A.** Hearts oxidative stress from control and Western diets-fed mice were measured by 4HNE staining. Representative image of 4HNE staining hearts from control **(B)** and Western **(C)** fed mice $n = 7-8$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$; two-way ANOVA followed by a Tukey's multiple comparison test. H9C2 rat cardiomyoblasts were plated and treated them with 100 μM IRE1 morpholino for 24 hrs. Then, cells were treated with or without 0.25 μM DOX. **D.** Cell viability was measured by fluorescent microscopy (BioTek Cytation, Agilent Technologies) or Cell Imaging Multi-Mode Reader, combining fluorescence and high contrast brightfield imaging with conventional multi-mode detection. **E-G.** Role of IRE1 in mitochondria respiration. A respirometry experiment was performed to measure the OCR which

is a measure of mitochondrial respiration on H9C2 cells. **E**. OCR measure, **F**, basal OCR, and **G**, maximal OCR. n=3; two-way ANOVA followed by a Tukey's multiple comparison test *p<0.05.

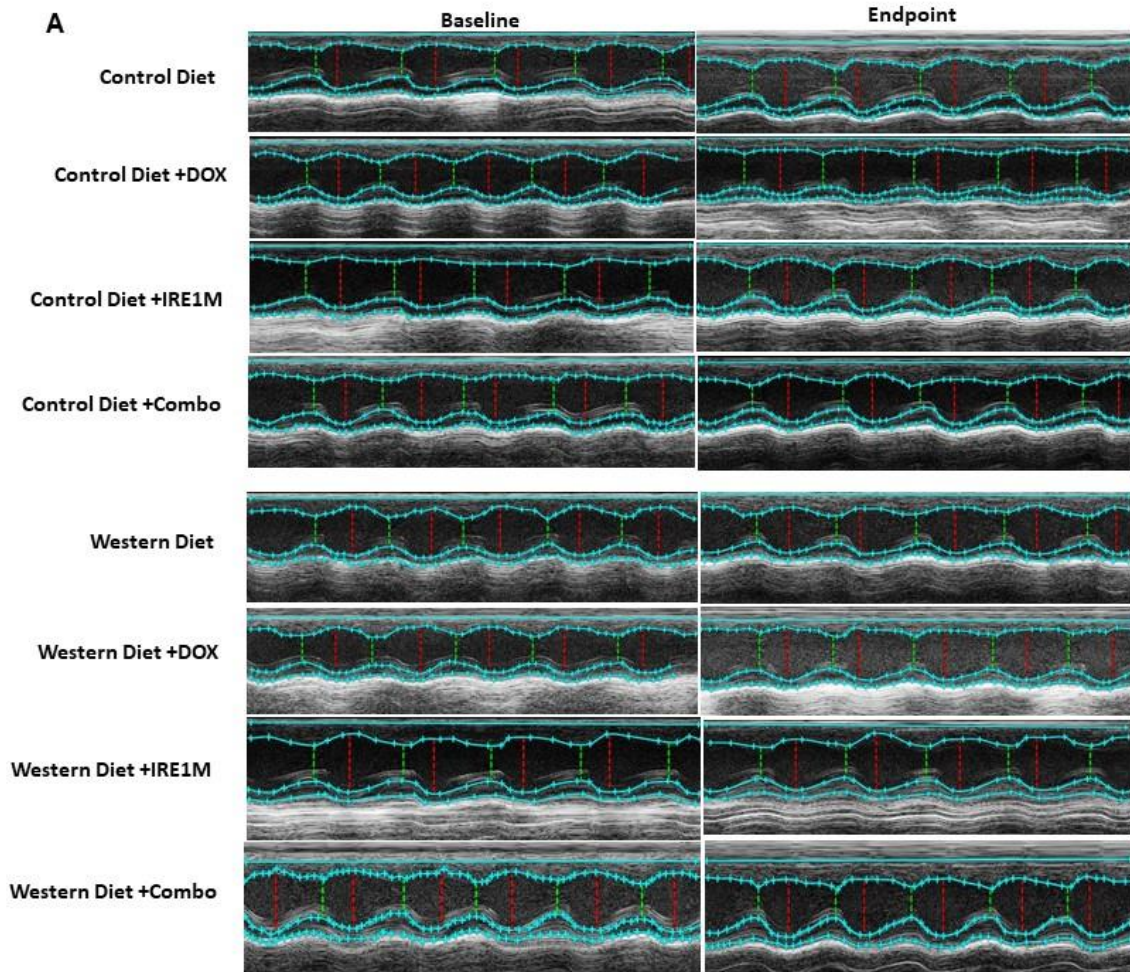
Supplemental Figures and Legends

Supplemental Figure S1.



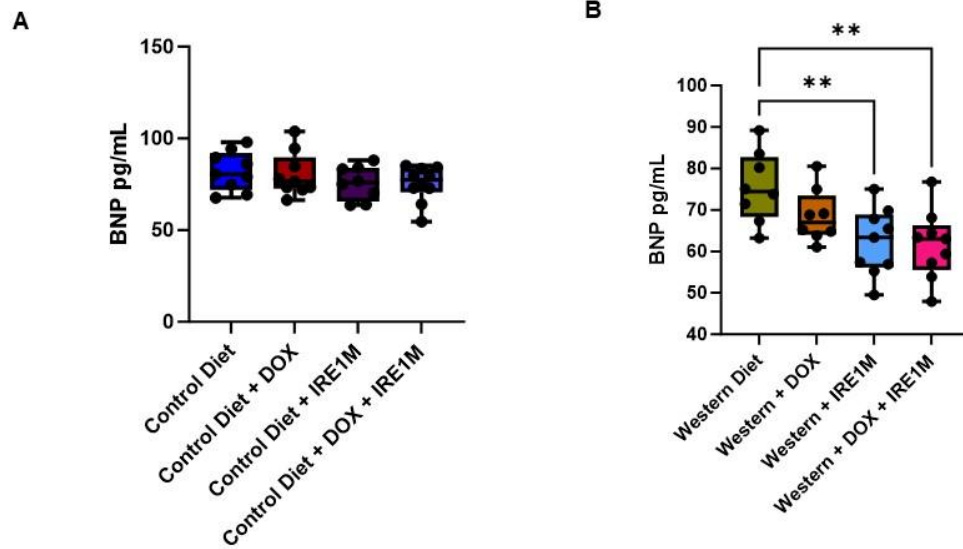
Supplemental Figure S1. IRE1 morpholino reduces tumor and heart IRE1 protein levels. IRE1 mouse anti-sense morpholino reduces IRE1 protein levels in tumors and hearts. **A-F.** Tumor and cardiac tissue were obtained from control and Western diet-fed mice, paraffin-embedded and sectioned. Sections of tumors (**A**) and hearts (**D**) were stained with DAB (IRE1 α). $n = 7-8$, $*p < 0.05$; two-way ANOVA followed by a Tukey's multiple comparison test. Representative image of IRE1 α staining 4T1 tumors (**B-C**) and hearts (**E-F**) from control and Western-fed mice.

Supplemental Figure S2.



Supplemental Figure S2. IRE1 blockade modules cardiac dysfunction. A. Representative image of M-mode tracing using Vevo LAZR ultrasound in mice consuming control and Western diets at baseline and after doxorubicin (DOX) chemotherapy.

Supplemental Figure S3.



Supplemental Figure S3. Plasma cardiac damage marker BNP is not regulated by DOX. Plasma levels of the cardiac biomarker B-Type Natriuretic Peptide (BNP) in Control (A) and Western (B) diets fed mice n=8-10. *p<0.05; one-way ANOVA followed by Tukey's multiple comparison test.

CHAPTER III

General Discussion

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General Discussion

Cancer treatment is a challenging process for every patient. Doxorubicin (DOX) is a highly effective chemotherapeutic agent; however, it can result in severe cardiac dysfunction and heart failure due to mitochondrial dysfunction, oxidative stress, apoptosis, calcium dysregulation, and other possible mechanisms which could be detrimental to the long-term quality of life in all cancer survivors. It is well known that cardiovascular disease (CVD) is the leading cause of death worldwide and in the United States among women and men [1] [2]. Due to the clinical benefit of chemotherapy, we found an important new strategy that decreases or ameliorates the cardiotoxicity side effects from DOX, which does not interfere with the anti-cancer effects of cytotoxic therapy by targeting IRE1. IRE1 is an arm of the unfolded protein response (UPR) activated when unfolded or misfolded proteins accumulate within the lumen of the ER. We found that targeting IRE1 in TNBC tumors in high-fat diet-fed mice reduced tumor growth by increasing oxidative stress and apoptosis. Moreover, IRE1 blockade may prevent metastasis formation in high-fat diet-fed mice. Furthermore, targeting IRE1 prevents chemotherapy-mediated cardiotoxicity in low and high-fat diets by reducing oxidative stress and apoptosis and increasing mitochondrial function.

The purpose of the studies reported in this thesis was to determine whether targeting IRE1 signaling enhances chemotherapy-mediated tumor killing while preventing chemotherapy-related cardiac toxicity in obese and non-obese preclinical breast cancer models. Our work demonstrates a novel strategy to improve cytotoxic chemotherapy of triple-negative breast cancer by blocking IRE1

and brings a novel approach to identifying better treatment options for TNBC patients to improve overall survival and quality of life.

IRE1 modules breast cancer proliferation

Breast cancer is characterized by rapid development and progression due to many factors such as hypoxia, autophagy, cell cycle proteins, signal transduction pathways, altered cell metabolism, hormone signaling, tumor microenvironment, EMT, and other factors [3]. It is well-known that cancer cells are exposed to a variety of stressors, which can lead to perturbed protein folding in the endoplasmic reticulum (ER); when unfolding protein accumulates, Glucose-regulated protein 78 (GRP78) is released to correctly fold the protein allowing the UPR signaling pathway activated. PKR-like endoplasmic reticulum kinase (*EIF2AK3*; PERK), activating transcription factor 6 (*ATF6*), and inositol-requiring enzyme 1 (*ERN1*; IRE1) are the three arms of the UPR. UPR signaling is upregulated in many different types of cancers, including breast cancer [4] [5] [6] [7] [8] [9]. It has been demonstrated that chronic UPR signaling may contribute to the hallmarks of breast cancer as well as metastatic progression and therapy resistance [10] [11] [12]. UPR is usually inactive in non-cancer cells and active in cancer cells; then, cancer finds a way to use this pathway to promote survival. Therefore, the UPR signaling pathway is a strong candidate for developing novel breast cancer treatments.

The main arm of the unfolded protein response (UPR), IRE1, has been involved in tumor initiation and progression, although the exact mechanism

remains unclear. It is very well known that IRE1/XBP1 protein and mRNA levels are upregulated in breast cancer [13] [14] [15]. Also, previous studies demonstrate that in patients with TNBC, high expression of IRE1 signaling is associated with poor prognosis [11]. Consequently, IRE1 is a strong candidate for a novel target therapeutic option for breast cancer. Research showed that targeting IRE1 signaling can reduce tumor burden [15] [16]. In conjunction with these published findings, we found that targeting IRE1 (using an anti-sense morpholino, which is a molecule that inhibits translation of the IRE1 protein) in our TNBC tumor-bearing mice significantly reduced tumor proliferation by reducing tumor volume, tumor weight, and Ki67 (a marker of proliferation) with or without chemotherapy (DOX).

Therapeutic strategies to target the IRE1 signaling pathway

IRE1 represents a potential therapeutic target for several diseases, such as cancer and cardiovascular injury associated with ER stress. IRE1 has two isoforms which are encoded by different genes in humans, such as IRE1 α (*ERN1*) and IRE1 β (*ERN2*) [17]. IRE1 α expressed everywhere, although IRE1 β expression is limited to epithelial cells of the gastrointestinal tract and lungs [17] [18] [19]. The molecular structure of IRE1 consists of two main regions (luminal and transmembrane or cytoplasmic region). The luminal region contains the N-terminus with an 18 amino acid signal sequence (SS) and the N-terminal luminal domain (NLD), which consists of three main β -sheets that form a triangular plate [20]. The cytoplasmic region contains the serine/threonine kinase (S/T kinase) and endoribonuclease (RNase) domains, which consist primarily of α helices, and the

RNase domain extends to the C terminus of the IRE1 α protein [20]. Given that the functional activities of IRE1 α as a serine/threonine kinase and endoribonuclease mediate the endoplasmic reticulum stress [17] [20]. There are different options to target the IRE1 signaling pathway depending on the molecular structure of IRE1 **(Table 1)** [21] [22]. In general, the effects of RNase domain inhibition cause attenuation in XBP1 splicing, block RIDD activity, allow phosphorylation, unaffected oligomerization, and conformation-independent. However, the effects of kinase domain inhibition cause blocked phosphorylate, different impacts on RNase activity (activation or inhibition), disrupted oligomerization, and conformation-dependent [20].

Table 1. Inhibition of the IRE1α cytoplasmic region. Review in [20].		
Inhibitors	Domain	
APY29	Kinase	Competitive ATP [23] widely used in drug development, but it is toxic at low concentrations, which limits its application in different models [24].
Sunitinib	Kinase	FDA-approved anticancer drug that blocks the autophosphorylation activity of IRE1 α and, as a type I pharmacophore, is considered a potent activator of IRE1 RNase activity [23] [25] [26].

4μ8C	RNase	Selectively binds in the RNase catalytic abridged and blocks substrate access to the active site of IRE1, consequently inhibiting either XBP1 splicing or RIDD [27]. Also, 4μ8C should be carefully used in research aimed at IRE1α inhibition due to its unfavorable pharmacokinetic [28] [29]
IRE1 alpha kinase-IN-2 (C ₂₇ H ₂₃ F ₃ N ₆ O)	Kinase	Second class of kinase inhibitors, which can inhibit RNase activity via interaction with the ATP-binding site [23].
HAA	RNase	Selectively target IRE1α RNase function, inhibiting XBP1 splicing and RIDD activity in the cellular context [30].
KIRA analogs	Kinase	ATP-competitive, imidazopyridine-based inhibitors that inhibit RNase activity by allosteric interaction with the kinase domain [31] [32] [33] [34] [35].
MKC inhibitors	RNase	Reduced IRE1α activity and decreased levels of XBP1s [36] [37] [38].
IPA	Kinase	IPA activates the RNase domain by interacting with the ATP binding site,

		which predisposes the kinase domain to oligomerize [24] [39].
STF-083010	RNase	It inhibits IRE1 endonuclease activity without affecting its kinase activity after endoplasmic reticulum stress [40] [41].
B-109	RNase	Highly selective RNase inhibitor. It was recently developed to improve the cellular and in vivo efficacy, bioavailability, and pharmacokinetics of the existing inhibitors [42] [43]
Salicylaldehydes	RNase	Inhibit the site-specific cleavage of several mini-XBP1 SL RNAs as the substrates of IRE1 RNase in a dose-dependent manner [44].
Toyocamycin	RNase	Agent derived from <i>Actinomycete</i> strains that selectively inhibit ER stress-mediated cleavage of XBP1 mRNA without affecting IRE1 phosphorylation [45].

There are several limitations of mostly all tested IRE1 inhibitors due to their unfavorable pharmacokinetic or possible side effects. Our studies used an anti-sense morpholino to target IRE1 protein expression. Morpholinos are anti-sense

oligomers that contain 15 to 25 nucleotides in length [46]. Morpholinos effectively bind to target mRNA and block translation, thereby transiently knocking down expression. There are several areas of research where morpholinos are used: 1) to study the function of genes during embryonic development [47], 2) to investigate the expression and regulation of genes, 3) to study the role of specific genes in neuronal development, synaptic plasticity, and neuronal function [48], 4) to evaluate the efficacy and safety of potential therapeutic targets [49] and others.

Consequently, we design to use morpholinos because of their versatility, stability, and ability to selectively modulate gene expression, making them valuable tools in understanding biological processes and developing potential disease treatments [50]. Moreover, morpholinos are generally considered non-toxic, making them appropriate for use in various disease models and will be essential for clinical translation. In our tumor-bearing mice, mice were treated with 1 x weekly I.P. 30 μ M IRE1 mouse anti-sense morpholino (targets host (cardiac) and tumor) for 3 subsequent weeks. We showed by IHC staining that IRE1 morpholino (IRE1M) reduced tumor and heart IRE1 protein levels. IRE1M has the potential to be used in the clinic as morpholinos are available for the treatment of Duchenne muscular dystrophy [51] and are in clinical trials for the treatment of prostate cancer [52].

IRE1 blockade modulates apoptosis signaling in breast cancer

Breast cancer (BC) is a heterogeneous disease characterized by high incidence, high mortality, high recurrence rate, and poor prognosis [53] [54]. BC is divided into several subtypes based on molecular characteristics, and treatments are based on those molecular characteristics [55] [56] [57]. ER+ BC can be treated with tamoxifen and aromatase inhibitors. HER2+ BC can be treated with monoclonal antibodies against HER2, such as trastuzumab and TNBC, that do not express specific molecules and can be treated with cytotoxic chemotherapies **(Introduction Figure 2)**. The main goal of these treatments is to induce cell death, an important strategy to control the excessive proliferation of breast cancer cells through several mechanisms such as ROS generation, fibrosis, calcium deregulation, mitochondrial dysfunction, DNA damage, apoptosis, and others [58] [59]. Apoptosis is a programmed cell death that promotes cell growth and death by extracellular (extrinsic apoptosis) or intracellular (intrinsic apoptosis) signals [60]. In normal breast development, the balance between proliferation and apoptosis is highly controlled to maintain cell homeostasis [61]. However, when this balance is disrupted, it causes anti-apoptosis signaling pathway activation or pro-apoptosis signaling pathway deficiency, which can lead to uncontrolled cell proliferation, therapeutic resistance, and cancer cell recurrence [62] [63] [64]. This evidence suggests that apoptosis is a highly regulated and controlled process that promotes cancer cell death [65]. Therefore, inducing apoptosis is an important strategy to prevent excessive breast cancer cell proliferation.

We investigated the role of IRE1 blockade in the apoptosis signaling pathway in our TNBC tumor model. Tumor tissues were stained to evaluate caspase 3 cleavage to measure apoptosis signaling. We found that targeting IRE1 with morpholino in combination with chemotherapy increased tumor apoptosis (cleaved caspase 3) in mice consuming low- or high-fat diets. IRE1 has multiple functions, and it has been demonstrated that the unconventional splicing of XBP1, a key regulator of ER stress, controls cell proliferation and apoptosis. Also, IRE1 can activate MAPK kinase (ASK1/p-JNK), which triggers apoptosis [66] [67] [68]. It has been demonstrated that targeting IRE1 (the endonuclease activity) decreases cell proliferation and increases apoptosis in breast cancer [69].

To elucidate a mechanism on how targeting IRE1 increases apoptosis, we study how IRE1 blockade contributes to oxidative stress in tumor tissue. We stained the tumors for lipid peroxidation (4HNE), and we found that IRE1 blockade with morpholino significantly increased lipid peroxidation alone or combined with DOX in the high-fat diet-fed mice. These data suggest that IRE1 blockade may impair IRE1 signaling, which may disrupt the endoplasmic reticulum (ER) chaperones, the endoplasmic reticulum-associated protein degradation (ERAD) or their functions that affect cell survival and trigger apoptosis in TNBC cells. The IRE1 signaling pathway may be of interest for anticancer drug development strategies.

IRE1 blockade modulates tumor metastasis

Metastasis is one of the significant issues in clinical oncology. Metastasis occurs when secondary tumors develop at distant sites through the body relative to the primary tumor and account for approximately 90% of cancer-related deaths [70]. Several studies show that at the time of diagnosis, a primary tumor has already seeded thousands of cancer cells into distant organs [71] [72] [73]. However, metastatic colonization can take years to complete [74]. In general, numerous steps are involved in the formation of metastases. For example, cells from the primary tumor must increase and grow. Once tumors exceed 1 to 2 mm in diameter, vascularization within the tumor must occur. Cancer cells can then invade surrounding tissue and get into the circulation, where the cells must resist cell death due to detachment. After this, the cancer cells stop in specific organs, adhere to the cell wall, and extravasate outside the vascular system. As long as the microenvironment is suitable for the survival of cancer cells, the cell can proliferate and develop a metastatic lesion. The metastatic lesion can potentially develop into further metastases [70, 74]. Evidence showed that different primary tumors preferentially metastasize to distinct organs [75] [76]. For example, breast cancer metastases tend to develop in the lung, brain, liver, and bone [77].

Triple-negative breast cancer (TNBC) has a higher rate of metastasis compared to other subtypes [78]. Evidence suggests that at the time of diagnosis, 4-6% of TNBC patients will develop metastasis [79] [80]. However, within 6 years of diagnosis, up to 35% of patients with TNBC will develop metastasis. Most TNBC cells tend to disseminate before initial treatment and eradication of the primary

tumor. TNBC have poorer survival compared to other breast cancer subtypes due to their higher rates of relapse and metastasis [81]. Therefore, therapies that prevent metastatic disease progression are needed for patients with TNBC. There are several approaches to prevent the development of metastasis in patients with TNBC. One regimen involves adding a therapy in combination with the initial chemotherapy to reduce the likelihood of developing metastatic disease. Another approach involves therapeutic treatment after the standard of care but before the development or detection of metastasis. The last option consists of administering a therapeutic once the metastatic disease is detected. Aside from choosing the best timing for therapeutic administration, drugs can target different processes involved in metastatic progression. Because there are no effective therapeutics to target metastatic processes in TNBC, additional research is needed to develop better treatment options for patients with this disease. For these reasons, we study the role of IRE1 in TNBC tumor metastasis. We used a murine model of triple-negative breast cancer, 4T1 cells, which has been shown to metastasize to the lungs in less than 60 days [82]. We demonstrated that targeting IRE1 alone reduced lung lesions in mice fed a high-fat diet. Interestingly, we found that targeting IRE1 with morpholino in combination with chemotherapy reduces lung lesions in low-fat and high-fat diet-fed mice. This suggests that targeting IRE1, in the context of chemotherapy, plays a significant role in TNBC tumor progression and metastatic development. However, more research is needed to elucidate the mechanism of action on how IRE1 blockade reduces tumor metastasis in combination with chemotherapy.

There are several essential components involved in tumor progression and the development of metastasis, such as the tumor microenvironment, composed of the extracellular matrix (ECM), blood and lymphatic vascular network, and stromal cells [83] [84] [85] [86] [87] [88] [89] [90]. We could evaluate further as a future study. We propose to study how targeting IRE1 specifically modulates the tumor microenvironment, fibroblasts, and inflammatory cells. Fibroblasts are the most studied stromal cells in the tumor microenvironment. Activated fibroblasts, called myofibroblasts or cancer-associated fibroblasts (CAFs), remodel the ECM, induce angiogenesis, recruit inflammatory cells, and stimulate cancer cell proliferation [83]. When fibroblasts become activated, the cells secrete more ECM components and are more sensitive to extracellular signals [84]. Importantly, CAFs can secrete hepatocyte growth factor (HGF), transforming growth factor beta (TGF- β), and platelet-derived growth factor (PDGF) to promote invasion and metastatic progression, in addition to their ability to remodel the ECM. On the other hand, Inflammation promotes tumor progression by secreting inflammatory cytokines such as interleukin 6 (IL-6), tumor necrosis factor-alpha (TNF- α), interleukin 1 β (IL-1 β), and interleukin 8 (IL-8) which promote breast cancer progression [86] [87]. Cytokines play an important role in tumor metastasis. So, it will be essential to study them specially, TGF- β and IL-6, which promote epithelial-to-mesenchymal transition (EMT), a process in which cells gain migratory features crucial for metastatic progression.

IRE1 blockade prevents cardiac dysfunction from chemotherapy

Cancer and cardiovascular disease (CVD) are the leading causes of death worldwide. Even though they are considered separate diseases, it appears that they are highly connected and affect each other's outcome at multiple levels (inflammation and oxidative stress) [91] [92]. Cardiac diseases and cancer share similar risk factors such as genetic predisposition, smoking, obesity, hyperlipidemia, sedentary lifestyle, diabetes, aging, and others [92]. It is well known that chemotherapeutic agents can cause detrimental effects on the structure and function of the heart, a consequence that has profound clinical implications for patient treatment [93]. That's why improvements in breast cancer survival have led to identifying potential late effects of breast cancer treatment on the risk of dying from cardiovascular complications. Evidence indicates that women with breast cancer history are at increased risk for CVD and CVD-specific mortality than their cancer-free peers [94]. Our proposed study highlights an opportunity to address cardiotoxicity in survivorship issues and improve breast cancer patients' quality of life. We used DOX in our studies because it is a very effective chemotherapy. Still, at the same time, it is one of the most toxic chemotherapies used in adults and pediatric cancer patients. It has been demonstrated that DOX treatment in adult cancer patients is associated with larger hearts that are unable to pump enough blood due to either loss of contractility (HFrEF) or reduced LV chamber volume (HFpEF) [95]. Also, pediatric patients who received DOX in their treatment regimens experience long-term reductions in cardiac mass, resulting in a heart that is not large enough to produce sufficient cardiac output for their adult

body size, which can cause diastolic dysfunction [96] [97]. Although some treatments exist to mediate DOX cardiotoxicity, such as dexrazoxane (FDA-approved), their use is limited to only a subset of adult patients [98] [99] [100]. Also, Ang II, beta-blockers, and statins are safe in CVD; however, their efficacy on DOX cardiotoxicity is unclear. For that reason, mitigation of the cardiac effects of DOX remains a clinical problem because when cardiac dysfunction develops, the likelihood of complete recovery is reduced despite optimal treatment [101]. However, it should be noted that early intervention can stop the progression of damage and improve cardiac function.

To develop a new and proven strategy for DOX cardiotoxicity that does not reduce the efficacy of chemotherapy, we propose to target the IRE1 signaling pathway using an antisense morpholino considered non-toxic. As mentioned above, IRE1 α is the most fundamental ER stress sensor conserved in all eukaryotic cells. It has been suggested that the accumulation of misfolded proteins in the ER compartment may contribute to vascular and cardiac diseases [102] [103] [104] [105]. It is well known that several factors will interfere with the homeostasis of ER function and cause the accumulation of misfolded or unfolded proteins in the ER lumen. ER stress then triggers the unfolded protein response (UPR) that induces signal transduction events to reduce unfolded protein accumulation. However, if the stress is severe or prolonged, the ER also initiates apoptotic signaling [102] [103]. Under severe ER stress, IRE1 causes activation of the amino-terminal kinase c-Jun and cleavage of caspase-12, leading to cardiac cell death and ultimately leading to cardiac injury [103] [106]. Furthermore,

activation of IRE1 causes the activation of inflammatory factors associated with inflammation in cardiovascular tissue [107]. Target IRE1 protein may ameliorate cardiac dysfunction; it has been shown that targeting IRE1 reduces caspase-1 activity and IL-1 β secretion, which significantly reduces cardiovascular lesion formation [108]. We demonstrated that targeting IRE1 in combination with chemotherapy reduces cleaved caspase-3 (apoptosis). Moreover, in our studies, we measure cardiac function by measuring ejection fraction (EF) and fractional shortening (FS) using a VEVO LAZR ultrasound system in the M and B modes. This echocardiography was utilized to assess DOX-mediated cardiac morphometry. Motion mode (M-mode), a two-dimensional cross-sectional representation of the LV heart wall movement, was used to measure chamber diameter and wall thickness throughout the cardiac cycle. These measurements provide a representative snapshot or static moment in time of the structural characteristics of the dynamic heart. As expected, DOX reduced cardiac functions in mice consuming low-fat and high-fat diets. Targeting IRE1 prevents cardiac dysfunction from DOX treatments by increasing EF and FS in both diets. Also, we evaluated cardiac dysfunction markers such as cardiac troponin (Troponin I type 3) in the serum and found that targeting IRE1 reduced Troponin I type 3 in low-fat diet-fed mice but did not cause any changes in high-fat diet fed-mice. Also, targeting IRE1 reduced B-type natriuretic peptide (BNP) levels, a marker of left ventricular failure. Our data suggest that targeting IRE1 represents a novel strategy to prevent cardiac dysfunction through DOX treatment.

Anti-fibrotic effect of IRE1 blockade in Dox cardiotoxicity

One of the main effects of DOX cardiotoxicity is collagen deposition and fibrosis. In response to tissue injury, collagen deposition, and tissue fibrosis are normal cellular processes involved in the repair process. However, sustained fibrosis in various tissue pathologies leads to decreased organ function [109]. In cancer patients, perturbations in the makeup of the ECM by maladaptive collagen deposition play a significant role in DOX cardiotoxicity [110] [111] [112]. DOX has been shown to increase TGF- β 1, leading to profibrotic signaling, in H9C2 rat cardiomyocytes [113] and endothelial cells [114], as well as in murine models of Dox-induced heart failure [115]. Dox-induced collagen deposition in the extracellular space of the heart and vasculature creates resistance during cardiac contractility and lusitropy, an active energy/ATP-dependent process [116] [117].

Knowing this information, we studied the role of IRE1 signaling in cardiac fibrosis resulting from DOX treatment. We performed pic red staining in our heart tissues. Pic red is a strong anionic dye that stains collagen by reacting via its sulfonic acid groups with basic groups in the collagen molecule [118]. In our studies, staining with pic red showed that DOX increased pathological collagen deposition in the interstitium and coronary vessels in mice consuming low-fat and high-fat diets. However, targeting IRE1 with morpholino significantly reduced intestinal and perivascular fibrosis in both diets. ER stress and the UPR pathway enhance the activation of several markers that result in cardiac remodeling and fibrosis by activating proapoptotic pathways, inducing epithelial-mesenchymal transition, and promoting inflammatory responses [119] [120]. Evidence has

shown that IRE1/XBP1 can act downstream of calreticulin expression to induce cardiac fibrosis by activating the TGF β 1/Smad2/3 signaling pathway [121]. Furthermore, activation of the Sigma-1 receptor inhibited cardiac fibrosis by inhibiting the IRE1-XBP1 pathway [122]. Together with these findings, our data suggest that targeting prevents the maladaptive response to DOX exposure. IRE1 may represent a therapeutic target for cardiac fibrosis. However, more work is needed to define better the mechanisms by which IRE1 modulates cardiac fibrosis through DOX treatment.

IRE1 blockade modulates DOX-induced oxidative stress and mediates the metabolism of cardiac cells

Superoxide generated by Nox enzymes acquires an electron from membrane lipids to form an unstable lipid peroxide, which decomposes into various peroxidation products, including malondialdehyde (MDA), the significant aldehyde-derived artifact, and 4-hydroxynonenal (4-HNE), the primary membrane-derived product [123]. Numerous studies showed that DOX administration increased MDA and 4-HNE markers of lipid peroxidation [124] [125] [126] [127] [128] [129]. Our results agree with these studies, showing the increase in 4-HNE marker after DOX treatments in mice consuming low-fat and high-fat diets. Moreover, DOX treatments can target mitochondria, causing cardiomyocyte damage and leading to superoxide radical formation [130] [131] [132]. Therefore, DOX treatment will cause changes in cardiac cell metabolism due to oxidative stress that may contribute to heart failure or abnormal contractility and relaxation

[131] [133] [134] [135] [136]. Our studies found that targeting IRE1 using morpholino significantly reduced lipid peroxidation marker (4-HNE), suggesting that IRE1 blockade modulates oxidative stress in cardiac tissue after DOX treatment. Along with these findings, prolonged ER stress and activation of the UPR have been shown to impair Ca^{2+} handling and increase oxidative stress and cellular apoptosis, all of which contribute to the pathogenesis of heart disease [137]. On the other hand, to elucidate a cardio-protective mechanism, we study the role of IRE1 on mitochondrial respiration. Growing evidence demonstrated that UPR arms have a cardio-protective effect in cardiac injury (ischemic insults) [138] [139] [140] [141]. Interestingly, targeting IRE1 in combination with chemotherapy increases oxygen consumption rate (OCR) as a measure of mitochondrial respiration on H9C2 rat cardiomyoblasts. These data suggest that IRE1 blockade may enhance mitochondrial function during stress as a potential cardio-protective mechanism. Another possible cardio-protective mechanism we proposed to investigate is the role of IRE1/Nrf2, a mediator of the expression of an array of antioxidant genes [142]. IRE1 is a conserved regulator of the human Nrf2 antioxidant response. Nrf2 is activated by 1) NADPH oxidase ROS or mitochondria ROS, which causes IRE1 protein sulfenylation that activates p38 and eventually Nrf2, and 2) by KEAP1 [143]. Nrf2 activation promotes the transcription of what is typically thought of as cardio-protective genes (Hmox1 (Heme oxygenase-1), Nqo1 (NADPH quinone oxidase-1), Sxn1 (Sulfiredoxin-1) and Peroxiredoxin) [142] [144]. For example, Nrf2 positively regulates NADPH quinone oxidoreductase 1, which catalyzes the reduction and detoxification of highly reactive quinones [145]

and may play a key role in reducing DOX-induced superoxide generation [146]. DOX, as well as other targeted treatments, have been shown to directly modulate Nrf2 and the antioxidant response of cardiomyocytes through the activation of MAP kinases [147]. It is necessary to determine whether Nrf2 plays a role in the co-treatment of DOX and IRE1 in our mouse model cardiac tissues.

Cardio-oncology effect of IRE1 and CD47

Co-expression of UPR and CD47 is associated with poor prognosis in breast cancer. Our lab demonstrated for the first time that CD47 expression is regulated by UPR stress signaling and controls anti-tumor immune response [148]. CD47 is a widely expressed cell surface receptor that controls phagocytic activity by engaging its counter receptor, SIRP α , in macrophages. Also, autonomously or by binding to its ligand, Thrombospondin-1 CD47 activation can control cell fate under stress by loss of mitochondrial membrane potential, Increased permeability & swelling [149] [150] [151] [152]. It has been demonstrated that inhibition of GRP78 and the treatment with linoleic acid sensitized breast tumors to anti-estrogen therapy [8]. Also, these treatments modulate CD47. This effect was associated with increased macrophage infiltration. Moreover, it has been shown that targeting CD47 using anti-sense morpholinos increased tumor macrophage recruitment [152]. Also, it has been demonstrated that blockade of CD47 sensitizes tumors to death associated with local ionizing radiation while remarkably protecting soft tissues and bone marrow [152] [153]. In conjunction with these findings, we studied the role of CD47 in anthracycline-mediated tumor killing while preventing

chemotherapy-related cardiac toxicity (**APPENDIX**). It has been shown that DOX treatment increases CD47 expression in TNBC [154].

To evaluate the blockade of CD47 using anti-sense morpholinos in combination with chemotherapy in vivo (**APPENDIX**), we employed the 4T1 breast cancer model. We examined tumor and cardiac tissue viability as well as autophagy flux. Cardiac function was determined in vivo by ultrasound. We found that targeting CD47 enhanced the effect of doxorubicin chemotherapy in vivo by reducing tumor growth and metastatic spread. Also, blockade of CD47 enhanced immunogenic cell death in combination with doxorubicin. Interestingly, systemic suppression of CD47 protected cardiac tissue viability and function in mice treated with doxorubicin by the activation of autophagy. Also, CD47 blockade reduced doxorubicin-induced cardiac fibrosis, resulting in improved cardiac function in vivo. In conclusion, the differential responses observed with CD47 blockade are due to the autonomous activation of protective autophagy in normal tissue and the enhancement of immune cytotoxicity against cancer cells. Overall, our studies on IRE1 and CD47 promise a new avenue for anticancer drug development strategies in cardio-oncology.

Concluding Remarks

My dissertation research aimed to determine the impact of IRE1 on cancer chemotherapy-related-cardiotoxicity. Throughout our studies, we have determined that:

- (I) IRE1 plays an important role in tumor development. However, targeting IRE1 sensitizes TNBC tumors to anthracycline therapy to enhance its efficacy and further decrease tumor burden, revealing its potential to be combined with FDA-approved chemotherapy treatments.
- (II) IRE1 blockade enhances apoptosis and contributes to oxidative stress in TNBC tumor tissues.
- (III) IRE1 blockade may prevent metastatic disease
- (IV) Targeting IRE1 prevents cardiotoxicity from chemotherapy (DOX) by reducing cardiac injury markers, oxidative stress, cardiac cell death, and cardiac fibrosis.
- (V) As a cardioprotective mechanism, targeting IRE1 increases mitochondrial respiration during stress.
- (VI) The antitumor efficacy of targeting IRE1 and chemotherapy increased in the context of obesity. Our studies used control and Western diets, which are representative of those consumed by human patients.

Overall, our study highlights the importance of targeting the unfolded protein response pathway (IRE1) to prevent cardiac dysfunction associated with chemotherapy treatment in obese and non-obese patients with triple-negative breast cancer. Therefore, IRE1 has the potential to be a new target to use alone or in combination with cancer chemotherapy treatment in the clinic.

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APPENDIX

Combination of anthracyclines and anti-CD47 therapy inhibit invasive breast cancer growth while preventing cardiac toxicity by regulation of autophagy

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Combination of anthracyclines and anti-CD47 therapy inhibit invasive breast cancer growth while preventing cardiac toxicity by regulation of autophagy

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Abstract

Background: A perennial challenge in systemic cytotoxic cancer therapy is to eradicate primary tumors and metastatic disease while sparing normal tissue from off-target effects of chemotherapy. Anthracyclines such as doxorubicin are effective chemotherapeutic agents for which dosing is limited by development of cardiotoxicity. Our published evidence shows that targeting CD47 enhances radiation-induced growth delay of tumors while remarkably protecting soft tissues. The protection of cell viability observed with CD47 is mediated autonomously by activation of protective autophagy. However, whether CD47 protects cancer cells from cytotoxic chemotherapy is unknown.

Methods: We tested the effect of CD47 blockade on cancer cell survival using a 2-dimensional high-throughput cell proliferation assay in 4T1 breast cancer cell lines. To evaluate blockade of CD47 in combination with chemotherapy *in vivo* we employed the 4T1 breast cancer model and examined tumor and cardiac tissue viability as well as autophagic flux.

Results: Our high-throughput screen revealed that blockade of CD47 does not interfere with the cytotoxic activity of anthracyclines against 4T1 breast cancer cells. Targeting CD47 enhanced the effect of doxorubicin chemotherapy *in vivo* by reducing tumor growth and metastatic spread by activation of an anti-tumor innate immune response. Moreover, systemic suppression of CD47 protected cardiac tissue viability and function in mice treated with doxorubicin.

Conclusions: Our experiments indicate that the protective effects observed with CD47 blockade are mediated through upregulation of autophagic flux. However,

absence of CD47 in did not elicit a protective effect but it enhanced macrophage-mediated cancer cell cytolysis. Therefore, the differential responses observed with CD47 blockade are due to autonomous activation of protective autophagy in normal tissue and enhancement immune cytotoxicity against cancer cells.

Background

Due to advances in early cancer diagnosis and improvements in anti-neoplastic drugs, 20 million cancer survivors are expected by the year 2024 in the United States alone [1]. This is a positive milestone, but that success comes with serious adverse side effects causing secondary pathologies and limiting patient quality of life after anti-cancer therapy. Therefore, we need new drugs and combinatorial strategies that decrease or prevent off target effects but do not interfere with the anti-cancer effects of cytotoxic therapy. Breast cancer patients, particularly those diagnosed with triple-negative breast cancer (TNBC), which respond only to systemic cytotoxic chemotherapy, are susceptible to off-target effects of chemotherapeutic agents including anthracycline-based drugs. We previously demonstrated that blockade of CD47 sensitizes tumors to death associated with local ionizing radiation, but protects bone marrow and soft tissues [2, 3]. A hepatocellular carcinoma xenograft model suggested similar additive effects of CD47 blockade with doxorubicin chemotherapy [4] and another study found that doxorubicin treatment increases CD47 expression in TNBC [5].

CD47 is a widely expressed receptor that controls cell fate via two major functions: (1) when it interacts with SIRP α on phagocytic cells, a “don’t eat me”

signal results that limits phagocytic activity. (2) On binding to TSP1, CD47 transduces signals that alter cellular calcium, cyclic nucleotide, integrin, growth factor signaling and control cell, viability and resistance to stress [6]. This latter function is fundamental to understanding why targeting CD47 could provide therapeutic benefits in disease such as cardiovascular diseases and cancer. This duality in survival effects makes CD47 an ideal target for cancer therapy. However, the mechanisms resulting in the selective protection of nonmalignant tissues are not completely understood. Deficiency of CD47 protects T lymphocytes and primary endothelial cells from ionizing radiation through the activation of protective autophagy [3, 7]. Our group and others have attributed the anti-tumor effects of CD47 to enhancement of innate and adaptive immune system actions against the tumor [8][9]. However, potential cell-autonomous differences in the cytoprotective effects of CD47 blockade in normal versus cancer cells has not been examined, and is a critical question to address as agents targeting CD47 progress in human clinical trials.

We implemented a fully automated high throughput screen using a 2-dimensional cell proliferation assay, where 1912 approved or experimental drugs were tested against 4T1 breast cancer cells with or without CD47 antisense morpholino treatment. The objective was to determine that blocking CD47 would not interfere with the oncologic efficacy of cytotoxic cancer therapy. Our data shows that compounds in the anthracycline family fit this paradigm. Anthracyclines are used to treat several cancer types and are the standard approach for adjuvant treatment of breast cancer because they result in improved outcomes compared

to other modalities [10–13]. However, high cumulative doses of anthracyclines increase the risk of congestive heart failure by up to 26% [1]. Furthermore, although initial damage is acute, symptomatic manifestations occur long after chemotherapy.

In this study we report that the cytoprotective effect of blocking CD47 extends to cardiac tissue. Our data shows that blockade of CD47 in combination with anthracycline treatment reduced primary 4T1 tumor growth and prevented metastatic spread to the lungs. Moreover, cardiac tissue of mice administered with anthracycline alone showed increased cell death. However, heart tissue from mice administered the combination treatment showed protection from anthracycline mediated death. Our data suggest that the protective effect of CD47 are due to an increase in autophagy whereas the anti-tumor effects are associated to stimulation of macrophage mediated cancer cell cytotoxicity. As shown previously, CD47 blockade increased protective autophagy hearts deficient in CD47 treated with anthracycline just as we had observed before in our previous studies in T cells [7]. The sensitization of tumors to anthracycline therapy was associated with increases in danger associated proteins HMBG1 and Calreticulin. Overall, our data shows that CD47 generally protects against chemotherapy mediated cytotoxicity in normal cells, but this protective effect is lost in cancer cells and thus not compromising the oncologic efficacy of cytotoxic chemotherapy.

Methods

Cell Culture: 4T1 mouse breast cancer cells were a gift from Dr. Patricia Steeg [National Cancer Institute (NCI, National Institutes of Health (NIH) Bethesda, Maryland) H9C2 rat cardiac myoblast cells and Raw 264.7 murine macrophage cell lines were obtained from ATCC (CRL-1446, TIB-71 respectively. All cell lines were cultured in DMEM supplemented with 10% FBS penicillin/streptomycin, and glutamine kept at 37°C and 5% CO₂.

Quantitative High-Throughput Screen (qHTS). 4T1 breast cancer cells, were seeded in 1536-well plates at 500 cells per well, in 50 µl of medium. Anti-sense CD47 morpholino was administered at a dose of 10 µM and was incubated in the flask for 48 h prior to the screen. Compounds were added at multiple doses ranging from .8 nM to 46 µM. Cell viability was assessed after 48 hrs incubation at 37°C by adding 30 µl of CellTiter-Glo reagent (Promega) and measuring luminescence (RLU) after a 15 m incubation at 25 °C, with ViewLux (PerkinElmer) [14].

qHTS data analysis. Activity of the hits from the qHTS screen was analyzed as shown previously [15] [14]. Normalized data were Normalized fitted to 4-parameter concentration response curves (CRC) using a grid-based algorithm. Each CRC was then assigned a heuristic curve response class (CRC). Curve classes of -1.1, -1.2, -2.1 and -2.2 are considered highest quality hits; Classes of -1.3, -1.4, -2.3, -2.4 and -3 are inconclusive hits; and curve class values of 4 are inactive compounds. Additional parameters obtained from the qHTS fitting procedure and used for hit selection were the Maximum Response, which is the % activity at the

maximum concentration of compound tested (46 μ M) and the AC₅₀, which is obtained from the curve fit. Differential activity of each compound between the wild type and knock-down CD47 conditions in each cell line was determined by calculating a difference in the maximum response or logAC₅₀ for each compound, between the two CD47 conditions, in each cell.

In vivo 4T1 orthotopic breast cancer model: Female Balb/c mice were injected with 4T1 cells (1×10^5 cells) into the mammary fat pad to induce tumor growth using a 26 gauge needle. Once tumors reached an average of 100 mm³ mice (n=8) were randomized, and one group received weekly IP injections of antisense CD47 morpholino (CD47 M) at a volume of 500 μ L. Groups of control or morpholino-treated mice received 100 μ L saline or doxorubicin (DOX) intravenously 48 h after each CD47 M injection. Tumor size was measured every third day using a caliper, and wet weight of the tumors was determined at the end of the study.

LC3 Staining: H9C2 cardiac myoblast cells were transfected with a GFP-LC3 construct as shown previously [16] and treated 24 h after transfection. Images were sequentially acquired with Zeiss AIM software on a Zeiss LSM 510 confocal system and quantified blindly using Zen 2.3 sp1 software.

Histology: Tissue arrays from developed from tissues of human breast cancer patient sections analyzed in the Laboratory of Pathology National Cancer Institute, under approved protocol by the Institutional Review Board of the National Cancer Institute were immunostained as shown previously [9] using 1:250 B6H12 antibody. Immunoreactivity was examined through light microscopy. Mouse lungs

were harvested at the end of the study and subjected to Hematoxylin and Eosin stain to visualize tissue structure. Tumor lesions were quantified blindly by a pathologist under light microscopy.

Cytotoxicity Assays: 4T1 cells and H9C2 cells were plated at a density of 10,000 per well in a 96-well plate. 4T1 and H9C2 were transfected using 1 μ M Endoportor and 10 μ M CD47 morpholino. Twenty-four hours after transfection and plating, cells were treated with 1 μ g/mL DOX and were incubated for 48 h. Cytotoxicity was measured by LDH release detected using a colorimetric activity assay (Invitrogen) and spectrophotometer (Benchmark plus microplate spectrophotometer, Bio-Rad).

Real-Time Measurement of Macrophage Mediated Cytotoxicity. 4T1 breast cancer target cells were seeded into 16-well plates. Cell growth was dynamically monitored using xCelligence system (ACEA Biosciences) for 24 h. RAW 264.7 macrophages cells were activated for 24h at 37°C with 10 ng/ml of LPS in complete medium and also used at an effector/target ration ratio of 5:1. After addition of effector cells, measurements were automatically collected by the analyzer. Cell mediated cytolysis was calculated using xCELLigence software set to collect impendence data (reported as cell index).

Statistical Analysis: Data are presented as the mean $\pm$ standard error of the mean (SEM). Statistical differences were evaluated by Student's *t* test or one way analysis of variance (ANOVA) followed by Tukey post hoc test. Animal studies were evaluated by repeated measures ANOVA followed by Tukey post hoc test. The criterion for statistical significance was set at $p < 0.05$.

Results

Screening of anti-sense CD47 morpholino activity in combination with cytotoxic drugs. High CD47 expression is associated with decreased survival in many cancers including breast [17]. Analysis of TCGA breast cancers classified based on RNAseq data using the established PAM50 panel of 50 genes [18, 19] shows that CD47 mRNA is elevated in basal-like breast cancers relative to HER2 and luminal A and B subtypes (Figure 1A). Our examination of human breast cancer tissue sections shows that CD47 expression is elevated in cancerous lesions of patients diagnosed with invasive breast cancer when compared to normal breast tissue (Supplemental figure 1A).

We have previously shown that blockade of CD47 enhances radiation tumor growth delay while protecting normal tissue from death from ionizing radiation [9, 20]. Thus we wanted to know if CD47 blockade would also protect breast cancer cells from death associated with cytotoxic chemotherapy treatment and whether this additive activity extends to syngeneic tumors. Using a cell proliferation assay in a 1536-well microplate high throughput screening format, we screened a comprehensive library of approved and late stage development oncology drugs in combination with a CD47 antisense morpholino to find combinatorial strategies that are more cytotoxic against breast cancer cells. We used the 4T1 breast cancer cell line to identify whether blockade of CD47 would protect cancer cells from cytotoxic chemotherapy. The endpoint for the high throughput screen was cell viability assessed by measurement of cellular ATP using CellTiter Glo reagent. Compounds were tested at eleven doses ranging from 0.8 nM to 46 μ M. Our data

showed blocking CD47 decreased the anti-growth activity of doxorubicin (DOX) chemotherapy less than 2-fold (Figure 1B). Moreover, as observed in Figures 1 B-G, blockade of CD47 generally did not result in significant protection of breast cancer cells from cytotoxicity mediated by treatment with other members of the anthracycline family of drugs. In the 4T1 screen 3 out of 6 drug treatments showed a modest increase in IC50 with CD47 blockade a (Figures 1B, 1C and 1G, Table I). Treatment with DOX showed a 1.4 increase in IC50 (Figure 1B * $p < 0.01$), epirubicin a 2.0 fold IC50 increase (Figure 1C, * $p < 0.01$) and Mitotraxone a 3.0-fold increase IC50 increase (Figure 1G, * $p < 0.01$). However, t-test analysis of the Hill Slope value was not significant (differences between means, $963720 \pm 1.692e+006$, $p = 0.5995$).

To corroborate that blockade of CD47 would not protect cells from anthracycline cytotoxicity we examined cell cytotoxicity by measuring LDH release (Figure 1H) from 4T1 cells treated with DOX in the presence or absence of anti-sense morpholino. DOX treatment increased breast cancer cell cytotoxicity in the presence or absence of CD47 blockade (* $p < 0.05$). A similar result was obtained when treating MDA-MB-231 cells (Supplemental Figure 1B). Thus, our data indicates that blockade of CD47 does not directly limit the oncologic efficacy of anthracyclines.

CD47 is over expressed in human invasive breast cancer, and its blockade enhances anthracycline mediated tumor cytotoxicity in a murine model of breast cancer. We chose DOX as the lead compound for our studies since it is

widely used in the clinic particularly in patients presenting invasive breast cancer and triple negative breast cancer patients [21]. Moreover, clinical use of anthracyclines is limited due to its off target cytotoxic effects particularly in the cardiovascular system. We implanted 1×10^5 4T1 breast cancer cells in the fat pads of Balb/c mice and treated them with saline, DOX, CD47 morpholino (CD47M) or CD47M with DOX. Treatment with CD47M was done two days prior to each treatment with DOX and was repeated weekly for 30 days (Figure 2A). As observed in Figure 2B and 2C, tumors of mice treated with saline tripled in size. DOX treatment caused the expected reduction in tumor volume and weight, but the combination of CD47 blockade and DOX further reduced tumor mass by 50% (Figure 2B). We confirmed knockdown of CD47 with antisense morpholino by western blot using the CD47 antibody miap301 (Supplemental Figure 1C). Therefore, blockade of CD47 enhances the effects of DOX to reduce breast tumor growth in a syngeneic tumor model, indicating that CD47 potentiates anthracycline-mediated breast tumor therapy.

The 4T1 model of breast cancer is known to develop lung metastasis after cells are injected orthotopically in the host mouse [22]. At the end of the study we performed histopathological analysis of lung sections and observed that lungs of animals administered the combination treatment had a significant over 70% (* $p < 0.01$) reduction of metastatic lesions when compared to lungs of animals treated with saline (Figure 2D & 2E, $4.2 \pm .75$ saline vs. 1.125 ± 0.6391 CD47M+ Dox $n=8$). DOX alone caused over 50% reduction in metastasis when compared with saline alone ($4.2 \pm .75$ saline vs 2.000 ± 0.4629 doxorubicin alone, $n=8$).

CD47M monotherapy reduced metastasis by 46% when compared to saline alone. Notably only 25% of mice treated with the combination treatment developed metastatic lesions in the lungs when compared to 100% of mice treated with saline and 75% of both CD47M and DOX monotherapies (Figure 2E). This indicates that combination treatment of CD47M and DOX reduces metastatic spread, which suggests using this approach could improve clinical outcomes of breast cancer patients.

Blockade of CD47 enhances macrophage mediated cytotoxicity of breast cancer cell. The increased efficacy of combining DOX with CD47M in vivo contrasts with the weak cell-autonomous effects observed in vitro and suggested that a non-cell autonomous immune mechanism is involved. Since blockade of CD47 enhances macrophage-mediated cancer cell killing, we tested whether pre-treatment with DOX modulated macrophage cytolytic capacity. We plated 4T1 breast cancer cells in 16-well plates and performed dynamic monitoring of RAW 264.7 mouse macrophage-mediated cytotoxicity of 4T1 breast cancer cells by measuring cell impedance using the ACEA xCelligence system (Figure 3A). The ACEA system measures killing of adherent target tumor cells by a decrease in surface impedance, and is presented as a normalized cell index. Cell index is derived from the measured electrical impedance and is a quantitative measure of the number of viable adherent target cells in each well [23]. Therefore, a decreased cell index indicates loss of viable target tumor cells. This is an established quantitative real-time method for effector cell mediated cytotoxicity against

adherent target cells [9, 23]. We pretreated 4T1 breast cancer cells with anti-CD47 morpholino for 24h and DOX for 4h the following day then proceeded to remove the media and washed twice with new media and plated a suspension of macrophages at a 1:5 effector target ratio. As observed in figure 3A DOX pre-treatment reduced cell viability as expected from control (Figures 3B-C). Pre-treatment with DOX enhanced macrophage mediated cytotoxicity when compared to effector target pairs alone. Blockade of CD47 enhanced macrophage mediated killing over untreated effector target pairs around 15h (Figure 3B) but no difference was observed at 24h (Figure 3C). However, combination of CD47 blockade and DOX pre-treatment significantly enhanced macrophage mediated killing of cancer cells by reducing cell viability 5-fold (* $p < 0.001$).

This suggested that the enhancement observed in tumor killing in vivo by CD47 blockade is mediated in part by the macrophage mediated cytotoxicity against cancer cells. To determine whether CD47 regulated macrophage infiltration in vivo we stained tumor tissue sections with a fluorescent antibody to CD68 and DAPI to denote nuclei (Figure 3D). Our data shows that blocking CD47 in the presence or absence of DOX treatment increases macrophage infiltration by over 40% (* $p < 0.05$) when compared to DOX treatment alone (Figures 3D, 3E). DOX treatment showed an approximately 50% (* $p < 0.05$) decrease in macrophage infiltration when compared to saline treated animals (Figures 3D, 3E). The increase in macrophage infiltration was significant with CD47 therapy alone when compared to saline treated animals (Figures 3D, 3E, * $p < 0.05$). Assessment of HMBG1, which facilitates macrophage mediated cell clearance, was increased by over 10-fold in

the combination treatment when compared to control (Figure 3F). Single DOX and CD47M treatments increased HMBG1 2-fold ($*p<0.05$) and 3-fold ($*p<0.05$), respectively. Moreover, examination of calreticulin protein expression which facilitates the clearance of cancer cells by engaging its receptor on phagocytic cells [24] was increased by 30% ($*p<0.05$) with CD47 treatment alone or and over 50% ($*p<0.05$) with combination with DOX in tumor lysates from in vivo treated mice (Figure 3G-3H). This suggests that the enhanced reduction in tumor growth with the DOX and anti-sense CD47 treatment is mediated in part by activation of an immunogenic cell death pathway.

Blockade of CD47 prevents anthracycline-mediated cardiac cell and tissue toxicity. Anthracyclines are used to treat several cancer types and are the standard approach for adjuvant treatment of breast cancer because they result in improved outcomes compared to other modalities [10–13]. However, high cumulative doses of anthracyclines compromise cardiovascular tissue causing the onset of congestive heart failure in patients [1]. To determine whether targeting CD47 may protect cardiac tissue from acute anthracycline associated death, we excised hearts of mice after one round of weekly DOX treatment in the presence or absence of prior treatment with CD47M. Sections were paraffin-embedded and subjected to TUNEL staining to detect cell death and were stained with wheat germ agglutinin to demark cardiomyocytes. Hearts of mice treated with DOX showed over a 15-fold increase in TUNEL staining when compared to saline treated mice (Figure 4A, 4B). CD47 blockade in combination with DOX prevented the increase

in TUNEL positive nuclei when compared to DOX alone (Figure 4A, 4B). Since we have previously shown that blockade of CD47 increases autophagy as a mechanism to prevent death from ionizing radiation[16] we transfected H9C2 rat cardiac myoblasts with anti-sense morpholino to CD47 and treated with DOX to determine autophagy is regulated as a protective mechanism from cytotoxicity associated with DOX. Transfection of GFP-LC3 construct into cardiac myoblast shows that LC3 puncta is increased by over 50% (* $p < 0.001$) with CD47 treatment in the presence or absence of DOX (Figure 4C, 4D). Suggesting that autophagy is upregulated as a consequence of DOX treatment. Moreover, DOX treatment did not change accumulation of SQSTM1/p62 in cardiac cells when compared to control and showed a reduction in LC3 lipidation when compared to the combination treatment. (Figure. 4E-4G). Blockade of CD47 reduced the expression SQSTM1/p62 alone by 80% (* $p < 0.01$) (Figure 4E, 4F and increased LC3 lipidation by 20 % over control (Figure 4E, 4G), however combination treatment reduced SQSTM1/p62 degradation by almost 75% and increased LC3 lipidation 3-fold (* $p < 0.01$) (Figure 4E-4G). This data indicates that the protective effect of CD47 blockade against DOX associated cytotoxicity is associated with an increase in autophagic flux.

To determine whether autophagy is necessary for the protective effect against death associated with DOX we cultured cardiac myoblasts in complete medium and treated with the autophagy inhibitor chloroquine (CQ). CQ elevates lysosomal pH, resulting in the inhibition autophagosome lysosome fusion[25]. Cardiac cells were treated with CQ 1h prior to DOX treatment and cell viability was

measured by LDH release. DOX increased cytotoxicity in cardiac myoblasts (Figure 4H * $p < 0.001$). However, CD47 blockade prevented the anthracycline-mediated cytotoxicity of cardiac myoblasts, demonstrating that the protective effect of CD47 blockade is cell autonomous. However, the protective effect of CD47 blockade from DOX associated cell cytotoxicity was lost following treatment with CQ. Thus, the observed chemo-protection from DOX treatment requires an autophagy mechanism.

We have previously demonstrated that the cytoprotection conferred by CD47 blockade in nonmalignant cells is mediated by the upregulation of Autophagy Related Genes (ATG's)[7]. Our data shows that targeting CD47 in combination with DOX increased ATG7 gene expression by over 50% (* $p < 0.05$) when compared to the rest of the treatment groups (Figure 4I, 4J). This serves as a further indication that autophagy is regulated as a protective mechanism with blockade of CD47. siRNA mediated silencing of ATG7 did not affect cardiac cell cytotoxicity associated DOX treatment. However, silencing of ATG7 reversed the protective effect of CD47 blockade against DOX cardiac cell cytotoxicity as it increased cytotoxicity over control and the combination of DOX and CD47M conditions (* $p < 0.05$, Figure 4K). This indicates that the cytoprotective effect of CD47 against DOX cardiac cell cytotoxicity is mediated by upregulation of the autophagy pathway.

Deficiency of CD47 preserves cardiac function after treatment with DOX. The heart has restricted regenerative capability; cumulative doses of anthracyclines surpass the threshold of damage that triggers ventricular remodeling, causing cardiac injury and limiting function [26]. Since fibrosis can accumulate in cardiac tissue limiting contractility and function we measured cardiac fibrosis as measured by picrosirius red staining (Figure 5A). Examination of tissue sections showed that there is a 5-fold increase ($*p<0.01$) in Fibrosis after DOX treatment (Figure 5B) over saline. However, treatment with CD47 prevented the increase in fibrosis after DOX treatment. Therefore, we predicted that CD47 blockade will improve cardiac function inhibiting collagen deposition. To test this, we injected WT and Cd47^{-/-} mice with DOX I.V. and measured cardiovascular function using in vivo ultrasound with the Vevo LAZR system. M-Mode tracing with in vivo ultrasound (Figure 5C) shows no difference in cardiac function as measured by ejection fraction and fractional shortening between untreated WT and Cd47^{-/-} mice (Figure 5C top panels, 5D, 5E). However, treatment with DOX significantly reduced the ejection fraction by almost 40% and fractional shortening by 50% ($*p<0.05$). On the other hand, deficiency of CD47 preserved cardiac function after DOX treatment. These results indicate that CD47 blockade could be used as an adjuvant to prevent cardiac dysfunction in cancer patients receiving anthracycline chemotherapy and improve long-term cancer patient quality of life.

Discussion

One of the many challenges in cancer therapy is to maximally suppress tumor growth while preserving the integrity of normal tissue. We have previously demonstrated that blockade of CD47 enhances radiation growth delay in several syngeneic tumor models. In the same study we showed that blockade of CD47 spared bone marrow and soft tissues from death caused by IR [2]. Further studies show the protective effect was mediated by an increase in protective autophagy [3, 16]. A screen performed with 4T1 breast cancer cells demonstrated that blockade of CD47 did not interfere with the anticancer activity of most compounds (Figure 1B-1H). Since we have shown previously in several models that blockade of CD47 is protective against cytotoxic stress, our goal was to rigorously show that absence of CD47 would not interfere with the oncologic efficacy of chemotherapeutic compounds. Our results demonstrate that the anthracycline family of compounds fit these criteria. Our data confirms that DOX treatment is cytotoxic to cardiac myoblasts, but this toxicity is decreased by suppressing CD47, thus suggesting that targeting CD47 in combination with cytotoxic therapy may decrease the off target effects of chemotherapy. Because elevated CD47 expression on tumor cells is associated with increased cancer progression by limiting innate and adaptive antitumor immunity, targeting this receptor may also enhance the antitumor effects of chemotherapy by protecting tumor-associated immune cells but not the tumor cells.

Clinical studies generally show that increased expression of CD47 is associated with poor prognosis in several types of cancer, including breast cancer [27, 28]. A study with over 700 patients demonstrated that patients with high expression of CD47 in the bone marrow cells had worse disease free survival (DFS) when compared to those with low levels of CD47 expression [29]. The expression profile also correlated with SIRP α expression. However, the same study showed that CD47 was an independent prognostic factor, and increased expression was associated with reduced overall survival and DFS [30]. Prior data using RNAseq shows that CD47 expression is elevated in TNBC when compared to other breast cancer subtypes [31]. Consistent with previous publications our new data indicates that expression of CD47 is elevated in basal-like breast cancer, and clinical samples of invasive breast cancer. This suggests a link between CD47 expression and poor prognosis of breast cancer patients. Preclinical studies focus on how elevated CD47 expression allows cancer cells to escape innate immunosurveillance [32–34]. This reflects the normal homeostatic function of CD47 as a counter-receptor for SIRP α (Fig. 3) that blocks phagocytosis [35]. On the other hand, CD47 also serves as a signaling receptor for the matricellular protein thrombospondin-1 [36, 37]. This interaction regulates responses to stress, tissue perfusion, and angiogenesis. Relevant to cancer therapy, the thrombospondin-1 peptide 4N1, binds to CD47, and downregulates caspase-3 protecting thyroid carcinoma cells against cytotoxic agents including DOX [38]. Thus, elevated expression of CD47 could cause resistance to chemotherapy, while

also suggesting that inhibition of CD47 could be a pharmacological target to sensitize cancer cells to death.

Our published studies identified autophagy as a major target for CD47 signaling, to preserve cell and tissue viability during stress [3, 16]. Our data shows that autophagic flux is increased in cardiac cells deficient of CD47 after DOX treatment. Upregulation of autophagy has been demonstrated to protect against DOX associated cardiomyocyte cytotoxicity [39]. Moreover, DOX treatment of cardiomyocytes inhibits the autophagic flux, causing the accumulation of autophagosomes[40]. Consistent with these findings, DOX treatment causes accumulation of autophagosomes in cardiac myoblast evident by the clump pattern observed in figure 4C. which has been associated with accumulation of autophagosomes previously [41]. The inhibition of autophagic flux by DOX also explains why targeting with either CQ or knockdown with ATG7 (Figures 4H, 4K) does not further sensitize cardiac cells to death, which could be expected in the treatment of cancer cells.

Autophagy signaling can have divergent effects on tumors. Inhibition of autophagy can sensitize cancer cells to chemotherapy[42]. However, the promotion of autophagy can also promote cancer cell death, particularly in in vivo models, whereas a recent study reported that autophagy inhibition increased the efficacy of inhibiting the CD47/SIRP α interaction in a glioblastoma xenograft model [43]. However, whether CD47 blockade regulates autophagy in cancer cells is unknown. Our data shows that blockade of CD47 in 4T1 and MDA-MB-231 breast cancer cells does not interfere with DOX associated cytotoxicity and thus not

limiting its oncologic efficacy (Figures 1B-H and supplemental Figure 1A). Our data (not shown) suggests that the canonical pathway of autophagy is not regulated in cancer cells by blockade of CD47. MDA-MB-231 cells express high levels of LC3-II [25], suggesting that levels of autophagic flux may not play a cytoprotective role in these cancer cells.

Previous reports suggest DOX treatment increases autophagy signaling [44, 45]. However, this may be context dependent; DOX may have different cell viability effects in various cancer or normal cell types [40]. Duality of autophagic signaling is well known [46]. Autophagy is known as program cell death III as formation of autophagosomes were associated with apoptosis [47] and autophagy may supplement metabolic signaling to promote survival induce drug resistance [46, 48]. Therefore, these observations may be in part of the complexities of interpreting autophagy. Our goal here is to determine the regulation of autophagy at the time we observe cell death in the heart which was 24h after DOX treatment as shown by TUNEL staining (Figure 4A, 4B). While we can't rule out that other genes of the autophagy pathway are not regulated by CD47 in cancer, the canonical protective autophagy pathways seem to be regulated differently between malignant and non-transformed cells.

The differential sensitivity of normal and cancer cells to CD47 blockade may be influenced by many factors including metabolism differences in tissues, levels of ROS, inflammatory environment among others. However, the sensitization of cancer cells to chemotherapy by CD47 is due in part to the well-established effects of this receptor on anti-tumor immunosurveillance. Our data shows that

pretreatment with DOX and CD47 blockade significantly enhanced macrophage mediated killing of breast cancer cells (Figures 4A-4C). We have shown recently that sensitization of breast cancer cells to anti-estrogen therapy by targeting the Unfolded Protein Response (UPR) pathway is due in part to a reduction in CD47 expression and increase of the “eat me signals” calreticulin and high molecular group box 1 (HMGB1) [49, 50]. Therefore, regulation of these proteins in the absence of CD47 could account for the enhancement in the reduction of tumor growth over DOX alone.

DOX has been demonstrated to increase the release of “damage associated molecular patterns” (DAMPs) such as HMGB1, which stimulated immune cell clearance of B16 melanoma cells [51, 52]. Consistent with these results we observed increased levels of HMGB1 in DOX treated tumors when compared to saline treated controls, which could account for the enhanced macrophage-mediated cytotoxicity observed with DOX-pretreatment in vitro. Furthermore, blockade of CD47 in combination with DOX resulted in a 5-fold increase in HMGB1 over DOX alone, suggesting increased activation of an anti-tumor immune response over DOX consistent with the increased macrophage infiltration in the combination treatment (Figure 3D).

The clearance of cancer cells by CD47 blockade is limited by the expression of “eat me” signals such as calreticulin. Calreticulin facilitates clearance of cell by engaging its counter receptor LDL-receptor-related protein (LRP) on the phagocytic cell. Our data shows that in vivo blockade of CD47 resulted in an increase in calreticulin that was not impaired with DOX treatment. Dendritic cell

vaccination resulted in clearance of refractory lymphomas, which was associated with a decrease in CD47 and increase in calreticulin, HMGB1[53]. This strategy increase immunogenicity of tumors. Our data shows for the first time that targeting CD47 in combination with chemotherapy causes a similar regulation in breast cancer, indicating that the blockade of CD47 in combination with DOX results in activation of an immunogenic cells death pathway that results in enhanced tumor ablation in vivo. This combination treatment may also result in enhanced immunogenicity of breast cancer, which has been a limitation in observing meaningful benefits of immunotherapy in clinical treatment of breast cancer [54]. The immune microenvironment is complex and other immune cells contribute to anti-tumor immunosurveillance. Us and others have shown that CD47 enhances adaptive immune responses against cancer cells [9, 55], therefore the enhancement in tumor killing with CD47 blockade could be due to stimulation of other immune cell types.

CD47 was recently demonstrated to be under the transcriptional control of HIF-1 [56]. The low oxygen tension and the harsh conditions of the tumor microenvironment and expression levels of CD47 may be responsible for signaling effects in tumor and protection from cytotoxicity in tissues distant from the tumor. Cardiac tissue from our tumor bearing animals treated with DOX showed increased cell death, but this was inhibited when DOX was administered in combination with anti-sense CD47. The microenvironment in the heart is rich in oxygen and displays a different metabolic demand than the tumor. Therefore, the interplay of oxygen tension and metabolism may regulate CD47 expression as well as differential

responses of normal and malignant tissues to cytotoxic therapy. Further studies are warranted to determine if hypoxia and metabolism may play a role in the autonomous regulation of these differential responses to CD47 targeting.

Conclusions: Overall our studies indicate that blockade of CD47 enhances anti-tumor responses to chemotherapy while preserving viability and function of the cardiovascular system. Since agents targeting CD47 are currently in clinical trials, these experiments suggest new combinatorial strategies to improve responses of patients with invasive and triple negative breast cancer and the further examination of anti-CD47 therapy as an adjuvant to prevent secondary cardiovascular pathologies that arise as a consequence of chemotherapy in cancer survivors.

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List of Abbreviations

AC: Activity of Maximum Concentration

CD: Cluster of Differentiation

CD47 (-): CD47 Deficient

CD47M: CD47 Morpholino

CRC: Concentration Response Curves

DMEM: Dulbecco's Modified Eagle Medium

DOX: Doxorubicin

DAMPs: Damage Associated Molecular Patterns

HIF1: Hypoxia Inducible Factor 1

SIRP α : Signal Regulatory Protein alpha

UPR: Unfolded Protein Response

WT: Wild Type

Compliance with Ethical Standards**Conflict of Interest**

The authors of this manuscript have no conflicts of interest to report

Ethical approval

Experiments in presented in this manuscript comply with the current laws of the United States of America and institutional research integrity policies. Tissue arrays from developed from tissues of human breast cancer patient sections analyzed in the Laboratory of Pathology National Cancer Institute, under approved protocol by the Institutional Review Board of the National Cancer Institute. Animal studies and procedures were approved by the Internal Animal Care and Use Committee (IACUC) of the NIH Intramural Research Program protocol # LP-012 and the Wake Forest IACUC protocol # A16–085. Other datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Informed consent:

Not applicable

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Table 1. LogIC50 of WT and CD47 (-) treated with anthracyclines

4T1 Breast cancer cells screen LogIC50±SE		
Drug	WT (Log IC50)	CD47M (LogIC50)
Doxorubicin	$2.58 \pm 0.25 \times 10^{-7}$	$3.89 \pm 0.37 \times 10^{-7}$
Epirubicin	$1.69 \pm 0.18 \times 10^{-7}$	$3.98 \pm 0.43 \times 10^{-7}$
Pirarubicin	$1.02 \pm 0.19 \times 10^{-6}$	$1.74 \pm 0.25 \times 10^{-6}$
Alcarubicin	$3.83 \pm 1.03 \times 10^{-7}$	$4.35 \pm 1.33 \times 10^{-7}$
Idarubicin	$1.18 \pm 0.24 \times 10^{-7}$	$1.59 \pm 0.21 \times 10^{-7}$
Mitotraxone	$8.14 \pm 0.17 \times 10^{-7}$	$2.62 \pm 0.56 \times 10^{-6}$

Figures and Legends

Figure 1.

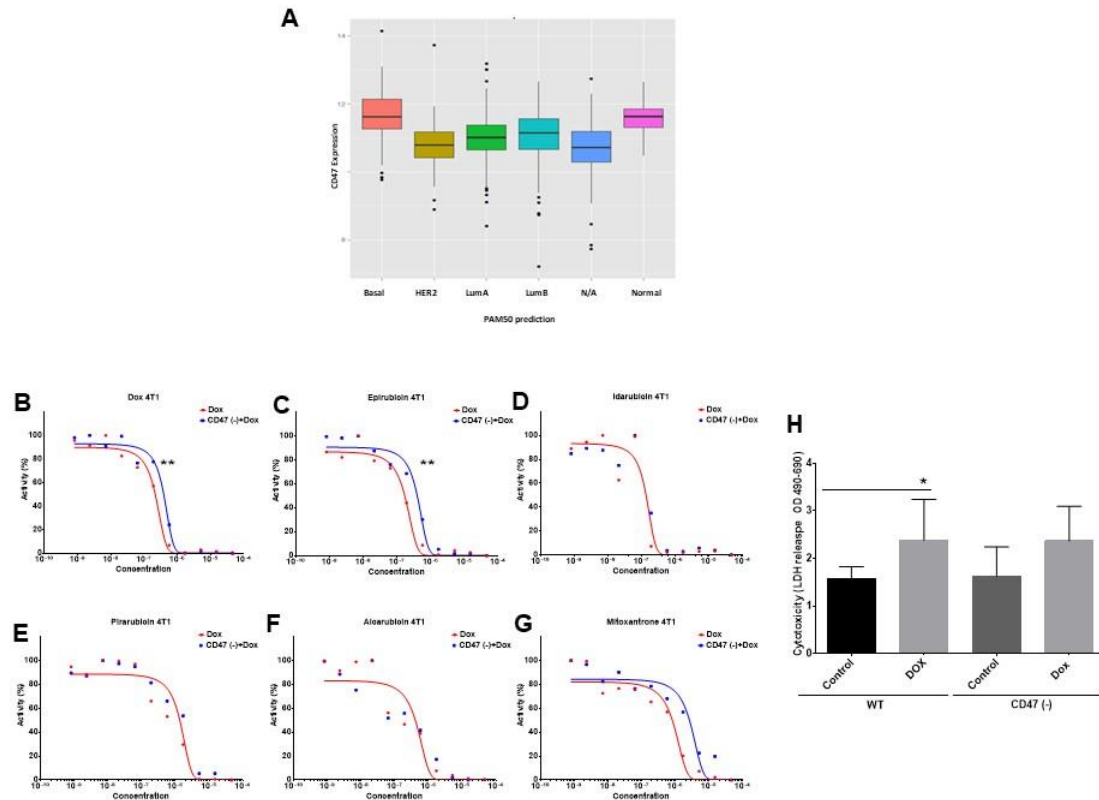


Figure 1. CD47 does not interfere with oncologic efficacy of anthracyclines.

(A) CD47 mRNA expression in TCGA breast cancers classified based on expression of the PAM50 panel of genes (B-G) High throughput screen measuring dose-responses for anthracycline effects on cell viability with or without CD47 blockade in 4T1 breast cancer cells. Twenty four hours after plating cells were subjected to automated robotic high throughput screen using by CellTiter-Glo® as

endpoint analysis. Curve regression analysis of data for anthracycline family compounds fitting concentration-response curves as (log(inhibitor) vs. normalized response) (* $p < 0.01$). (H) Measurement of DOX cytotoxicity in 4T1 breast cancer cells by LDH release (* $p < 0.05$).

Figure 2.

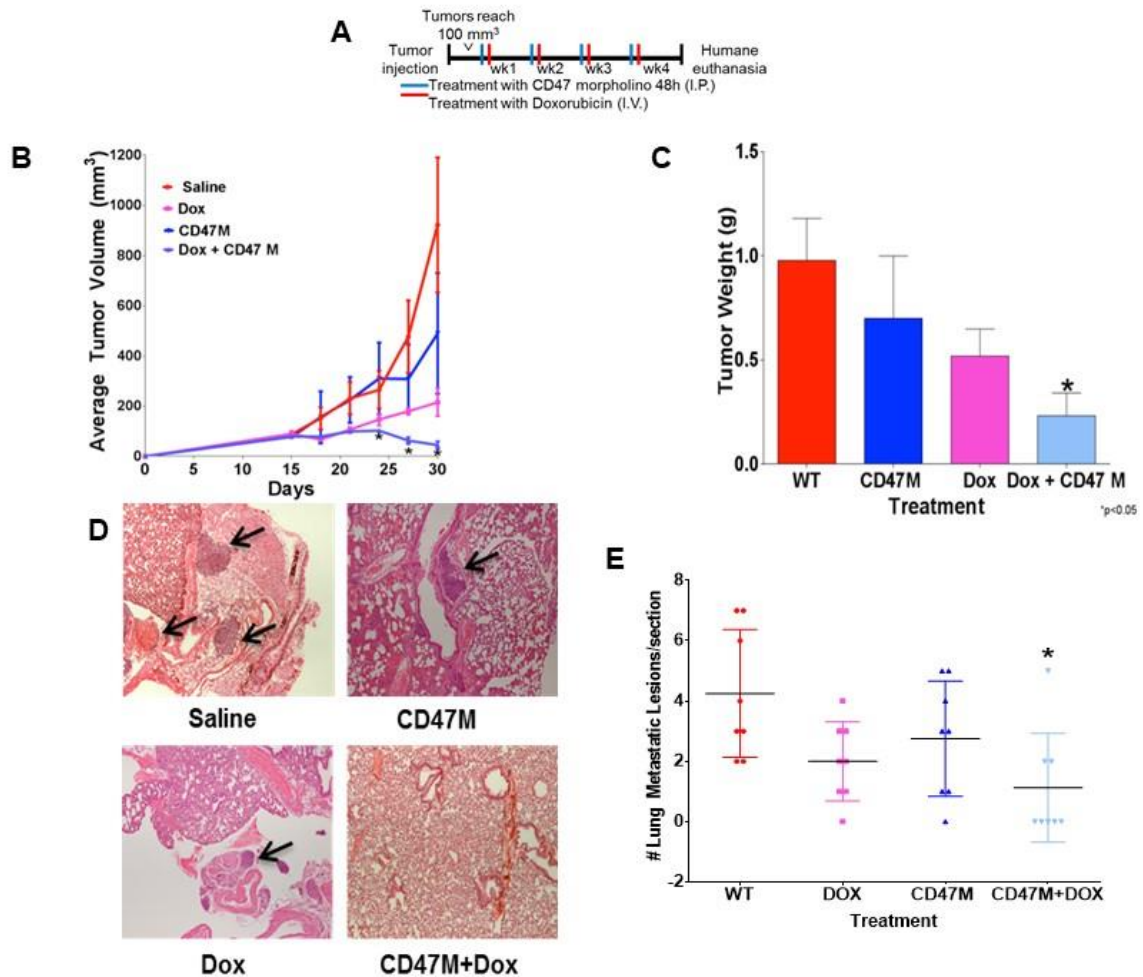


Figure 2. Blockade of CD47 enhances anti-tumor effects of DOX chemotherapy *in vivo*. (A) Schedule of treatment. (B) Balb/c mice were injected in the mammary fat pad with 4T1 mouse breast cancer cells. Tumor volume was measured with a caliper every three days and was calculated by the formula: $\text{volume} = W^2 \times L/2$, where W = shortest diameter and L = longest diameter (n=8,

* $p < 0.001$ repeated measures ANOVA, followed by Tukeys multiple comparison test. (C) Tumors were excised, and weights were recorded at the end of the study. (D, E). After 30 days animals were euthanized, and lungs were excised. Tissue sections were paraffin embedded and stained with Hematoxylin and Eosin to determine tissue structure. Tumor lesions were counted by the pathologist under bright field microscopy $N=8$, * $p < 0.05$.

Figure 3.

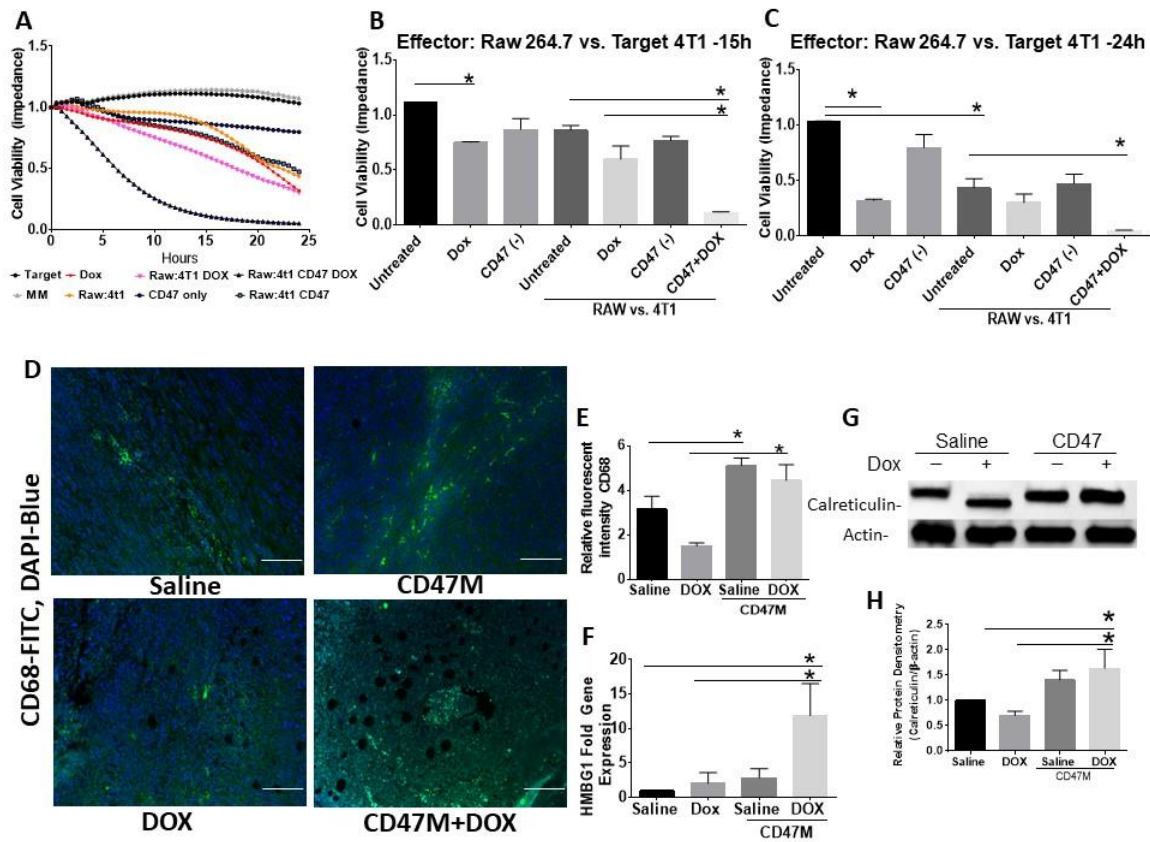


Figure 3. Blockade of CD47 enhances macrophage mediated clearance of breast cancer cells. (A) Macrophage mediated cytotoxicity was measured in 16-well plates equipped with electrodes to measure cell impedance, cell cytotoxicity was counted as changes in cells index (impedance) when cells migrated to bottom plate equipped with electrodes N=4 * $p < 0.05$ (B) cell viability at 15h and (C) 24 h post treatment (* $p < 0.01$). (D) Tumor tissue sections where stained with a fluorescent antibody to CD68 (FITC-green) to detect tissue infiltrating

macrophages and DAPI to denote nuclei (blue), (E) sections were quantified by examining fluorescence of CD68/DAPI over area of section at 20x under multispectral fluorescent imaging (Scale bar = 50 μ m). (F) HMGB1 expression was assessed by RT-PCR in tumor extracts (* $p < 0.05$), (G) calreticulin gene expression was assessed in lysates from in vivo tumor studies by Western Blot hybridization and (H) was quantified by densitometry (* $p < 0.05$).

Figure 4.

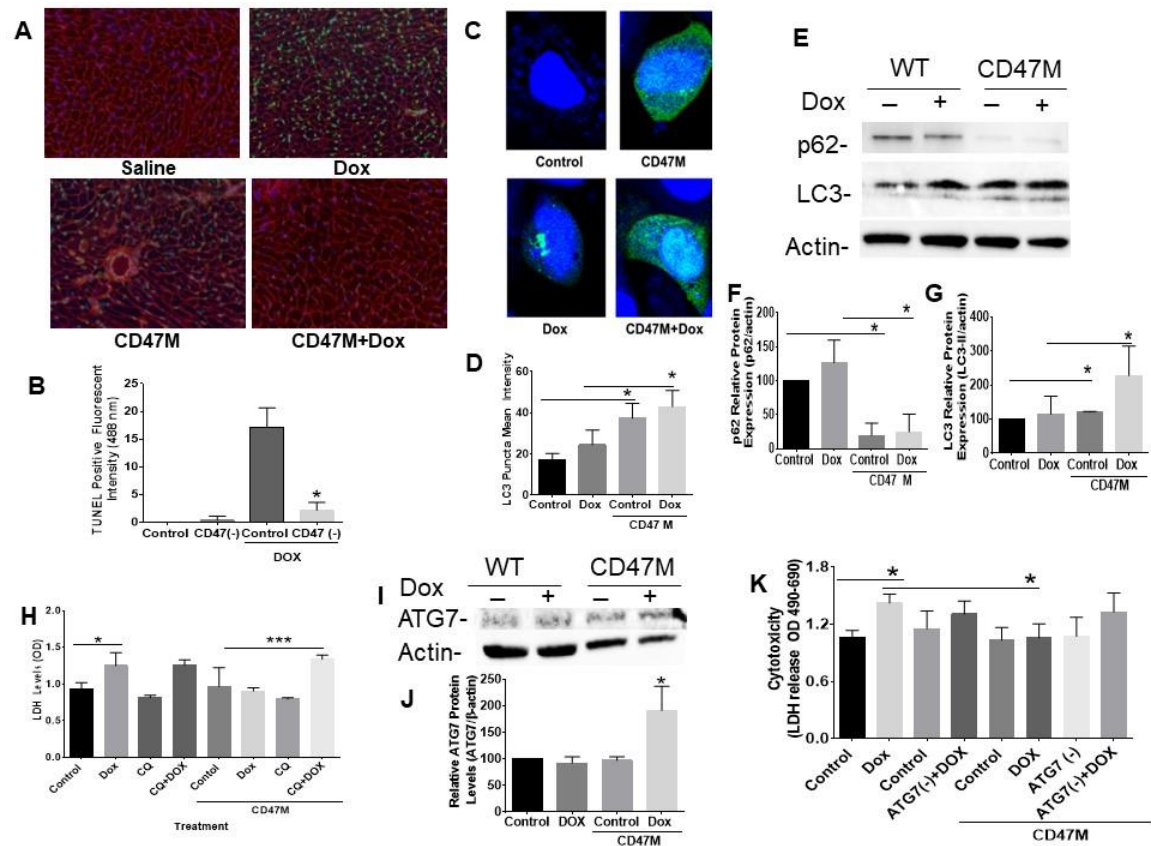


Figure 4. Blockade of CD47 protects cardiac myocytes and cardiac tissue from doxorubicin mediated toxicity. (A) Hearts of mice were excised after doxorubicin treatment, sections were paraffin embedded and tissue was stained with TUNEL and WGA (red) to detect apoptotic cardiac cells (40x, Scale bar= 100µm). Autophagic flux was measured by expression of SQSTM1/p62 and LC3 lipidation by Western Blot hybridization (B) and quantified (C, D n=4 *p<0.05). H9C2 cardiac myoblasts were treated with CD47M and or DOX and transfected with LC3-GFP LC3 puncta was determined by confocal microscopy (E) and

quantified using ZEN 2.3 SP1 software (F). H9C2 cardiac myoblast cells were plated and left untreated or administered CD47 Morpholino (CD47 M); 48 h after cells were treated with or without DOX in the presence or absence of 25 μ M chloroquine cells were incubated 48 h and LDH release was measured to determine cell cytotoxicity n=4 *p<0.001 (G).

Figure 5.

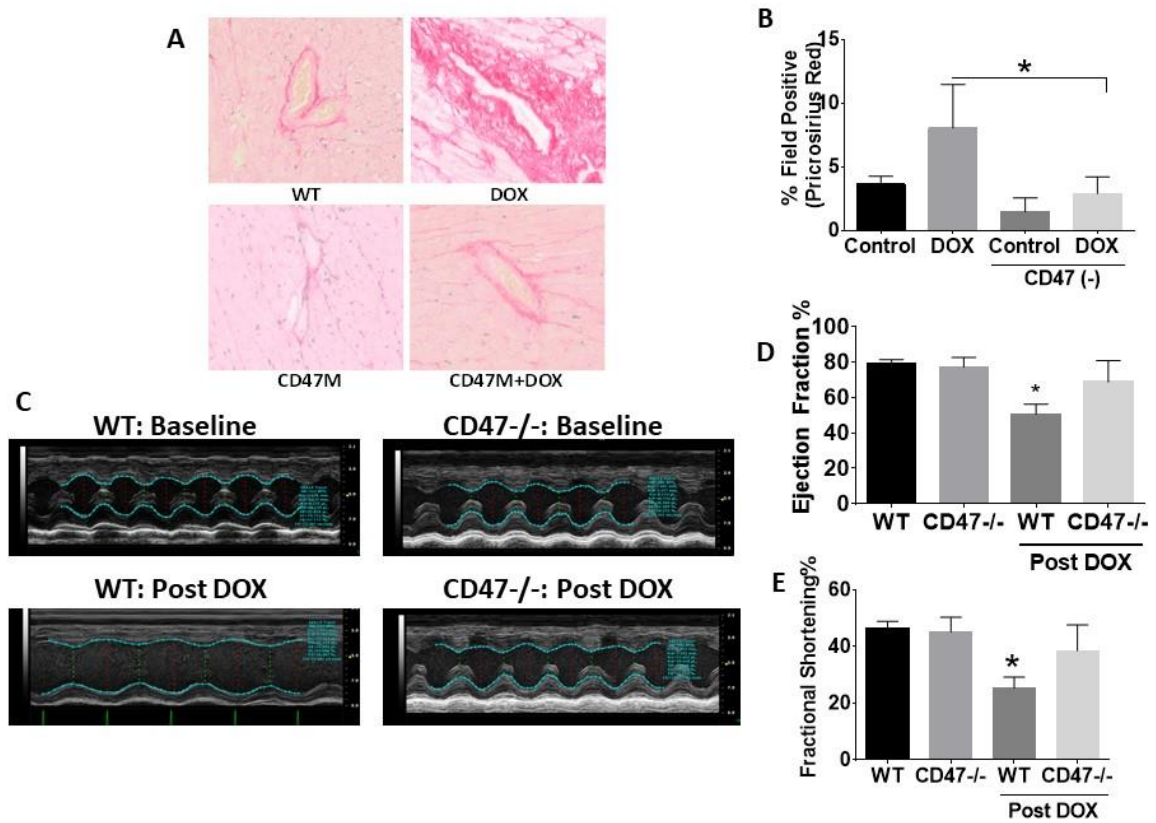
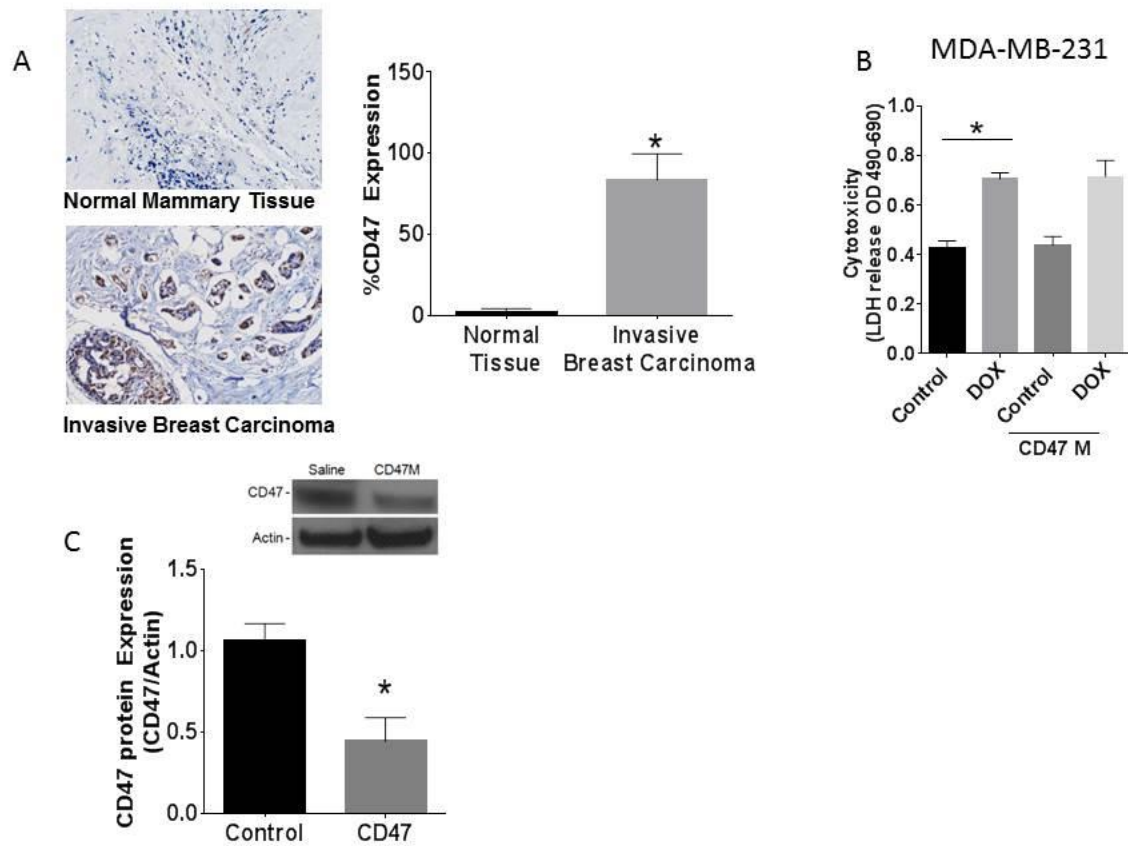


Figure 5. Deficiency of CD47 prevents DOX associated cardiac fibrosis and preserves cardiovascular function. Hearts of mice were excised at the end of the study and were paraffin embedded. Sections were stained with pricosirius red (A) and quantified using mantra inform pathology suite software (B) $n=5$ $*p<0.05$ (Scale bar= 50 μ m). Cardiovascular function was assessed using in vivo ultrasound with Vevo LAZR Photoacoustic Imaging System (C-E).

Supplementary Materials



Supplemental figure 1: (A) immunohistochemical staining of human mammary gland (top) and human invasive breast cancer (representative of 5 cases) with human antibody B6H12 (brown stain). (B) LDH release assay in MDA-MB-231 cells. (C) Efficiency of CD47 knock down using anti-sense morpholino treatment in tumors N=5, *p<0.05.

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hand, the excitement of translating basic concepts to the clinic.

February 2016 – Jun 2016

Breast Cancer and Vascular Research, Dr. David Soto Pantoja Lab, Hypertension and Vascular Research Center/Comprehensive Cancer Center/Surgery, Wake Forest University School of Medicine, Winston Salem, NC

Dr. David Soto Pantoja's lab focuses on investigating anti-breast cancer treatments and therapies. In these studies, we examine the role of CD47 in breast cancer resistance. In the lab I assisted Dr. David Soto Pantoja in identifying whether this differential relationship exists when using chemotherapy.

Summer 2015

Breast Cancer, Dr. Junjie Chen Lab, Department of Experimental Radiation Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX

I studied the effects of Histone Deacetylase (HDAC) Inhibitors of human Hippo-YAP pathway in triple negative breast cancer cell lines.

August, 2013 – January, 2016

Inflammatory Breast Cancer, Dr. Michelle Martinez-Montemayor Lab, Department of Biochemistry, Universidad Central del Caribe, School of Medicine, Bayamón, PR

I performed mechanistic studies to investigate the efficacy of natural therapeutics alone or in combination with conventional therapy using *in vitro* models of breast cancer (BC), including inflammatory breast cancer (IBC). Studies focused on cancer cell survival, protein synthesis, and proteomics through SILAC and mass spectrometry, and *in vivo* experimental studies.

Summer 2013

Independent Research – HAPMAP (IR) program, Dr. Michael Sadowsky's Lab., Department of Soil, Water and Climate, and The Biotechnology Institute, University of Minnesota, MN, USA

I studied the regulation of elevated levels of ethylene, which inhibit the association of rhizobia with legumes and prevent effective nitrogen fixation.

PROFESSIONAL WORK

Aug, 2012- Dec, 2013

Jan, 2014-Dec, 2015

Biology, General Chemistry and Math Instructor, Program of Educational Services, Inter American University of Puerto Rico, Bayamón, PR

I was responsible for tutoring undergraduate students in sciences. I mentored students that had difficulties in General Chemistry, Math and Biology courses in order to help them to overcome their challenges.

Summer 2009

Office Clerk, Department of Family Services, PR Government, Bayamón, PR

I was responsible of performing administrative duties such as filing documents, and receptionist related tasks.

PUBLICATIONS

Ramirez MU, Clear KYJ, Cornelius Z, Bawaneh A, **Feliz-Mosquea YR**, Wilson AS, Ruggiero AD, Cruz-Diaz N, Shi L, Kerr BA, Soto-Pantoja DR, Cook KL. Diet impacts triple-negative breast cancer growth, metastatic potential, chemotherapy responsiveness, and doxorubicin-mediated cardiac dysfunction. *Physiol Rep*. 2022 Apr;10(8):e15192. doi: 10.14814/phy2.15192. PMID: 35439354.

Feliz-Mosquea YR, Christensen AA, Wilson AS, Westwood B, Varagic J, Meléndez GC, Schwartz AL, Chen QR, Mathews Griner L, Guha R, Thomas CJ, Ferrer M, Merino MJ, Cook KL, Roberts DD, Soto-Pantoja DR. "Combination of anthracyclines and anti-CD47 therapy inhibit invasive breast cancer growth while preventing cardiac toxicity by regulation of autophagy". *Breast Cancer Res Treat*. 2018 Jul 28. PMID: 30056566.

Suárez-Arroyo IJ, Rios-Fuller TJ, **Feliz-Mosquea YR**, Lacourt-Ventura M, Leal-Alvarez DJ, Maldonado-Martinez G, Cubano LA, Martínez-Montemayor MM. "Ganoderma lucidum Combined with the EGFR Tyrosine Kinase Inhibitor, Erlotinib Synergize to Reduce Inflammatory Breast Cancer Progression". *J Cancer* 2016; 7(5):500-511. PMID: 26958085.

Suárez-Arroyo IJ, **Feliz-Mosquea YR**, Pérez-Laspiur J, Arju, R, Giashuddin S, Maldonado-Martinez G, Cubano LA, Schneider RJ, and Martínez-Montemayor M. M. "The proteome signature of the inflammatory breast cancer plasma membrane identifies novel molecular markers of disease". *Am J Cancer Res*. 2016 Aug 1;6(8):1720-40. eCollection 2016. PMID: 27648361.

John Michael Sipes, **Yismeilin R Feliz-Mosquea**, Katherine L. Cook, Gema Martin-Manso, Nicole L. Morris, Arunima Ghosh, Nancy J. Emenaker, David D. Roberts and David R. Soto-Pantoja. Thrombospondin-1 expression alters intestinal metabolism and microbiota during carcinogenesis in the *Apc^{Min/+}* mouse. **Submitted to Matrix Biology**

Yismeilin Feliz-Mosquea, Adam Wilson, David Soto-Pantoja, Katherine Cook. "Inositol-requiring enzyme-1 (IRE1) signaling impacts triple negative breast cancer chemotherapy sensitivity and prevents cardiac toxicity from

chemotherapy. **This manuscript is in preparation to be submitted in April 2024.**

Yismeilin Feliz-Mosquea, Kenysha Clear, Adam Wilson, Brian Westwood, David Soto-Pantoja, Katherine Cook. “UPR signaling pathway as a double edged sword: The unfolded protein response (UPR) differentially impacts breast tumor chemotherapy sensitivity and the development”. – **In preparation**

ORAL PRESENTATIONS

1. Blocking IRE1 Signaling Enhances Chemotherapy Response while Preventing Treatment-Related Cardiotoxicities in TNBC. Wake Forest University School of Medicine. Center for Redox Biology and Medicine. Fall 2023 Retreat, November 9, 2023.
2. Blocking Inositol-requiring enzyme-1 (IRE1) affects triple-negative breast cancer chemotherapy sensitivity and prevents chemotherapy-related cardiotoxicity. 2023 Ignite Conference in Wake Forest University School of Medicine, October 27, 2023.
3. Targeting IRE1 Signaling Enhances Chemotherapy Response while Preventing treatment-related cardiotoxicities in TNBC. Cancer Genetics and Metabolism (CGM) Annual Retreat on December 2, 2022.
4. Targeting inositol-requiring enzyme-1 (IRE1) affects triple-negative breast cancer chemotherapy sensitivity and prevents chemotherapy-related cardiotoxicity. 2022 SACNAS conference in San Juan, PR. October 27-29, 2022.
5. Targeting Inositol-requiring Enzyme-1 (IRE1) Signaling Impacts Triple-negative Breast Cancer Chemotherapy Sensitivity Preventing Chemotherapy-related Cardiotoxicity. Wake Forest University School of Medicine. Center for Redox Biology and Medicine. Spring 2022 Retreat, Thursday, May 12.
6. Inositol-requiring enzyme-1 (IRE1) signaling impacts triple negative breast cancer chemotherapy sensitivity and prevents cardiac toxicity from chemotherapy. American Society for Matrix Biology (ASMB) Biennial Meeting in St. Louis, MO. September 12-15, 2021.
7. Effect of Histone Deacetylase Inhibitors on Hippo-YAP pathway in triple negative breast cancer cell. 2nd Puerto Rico Cancer Research Meeting, San Juan PR. October 2, 2015.
8. Enhanced combinatorial response of *Ganoderma lucidum* and the HER2 tyrosine kinase inhibitor, Lapatinib in HER2+ inflammatory breast cancer cells. Cuarto Simposio de Investigación Sub-graduada de la Asociación Universitaria de Programas de Honor (AUPH), Gurabo PR. November 6, 2015.

POSTER PRESENTATIONS

1. **Feliz-Mosquea Y.R.**, Kenysha Clear, Adam Wilson, David Soto-Pantoja, Katherine Cook. Targeting inositol-requiring enzyme-1 (IRE1) affects triple-negative breast cancer chemotherapy sensitivity and prevents chemotherapy-related cardiotoxicity. 31st Annual Surgical Sciences Research Day November 16th, 2023
2. **Feliz-Mosquea Y.R.**, Kenysha Clear, Adam Wilson, David Soto-Pantoja, Katherine Cook. Inositol-requiring enzyme-1 (IRE1) blockade affects triple-negative breast cancer chemotherapy sensitivity and prevents chemotherapy-related cardiotoxicity. 30th Annual Surgical Sciences Residents' and Fellows' Research Day on November 15, 2022.
3. **Feliz-Mosquea Y.R.**, Kenysha Clear, Adam Wilson, David Soto-Pantoja, Katherine Cook. Inositol-requiring enzyme-1 (IRE1) blockade affects triple-negative breast cancer chemotherapy sensitivity and prevents chemotherapy-related cardiotoxicity. 12th Annual Women's Health Research Day. Wake Forest University School of Medicine. Bowman Gray Center for Medical Education. Tuesday, May 24, 2022.
4. **Feliz-Mosquea Y.R.**, Kenysha Clear, Adam Wilson, Brian Westwood, David Soto-Pantoja, Katherine Cook. Blocking IRE1 signaling pathway sensitizes triple-negative breast tumors to chemotherapy and prevents cardiac toxicities. Wake Forest Graduate School of Arts and Sciences 2022 Virtual Research Day. March 28, 2022.
5. **Feliz-Mosquea Y.R.**, Soto-Pantoja D.R., Clear K, Wilson A, Westwood B, and Cook K.L. Targeting Inositol-requiring enzyme-1 (IRE1) signaling impacts triple-negative breast cancer chemotherapy sensitivity and prevents chemotherapy-related cardiotoxicity. 29th Annual Surgical Residents' and Fellows' Research Day on November 4, 2021.
6. **Feliz-Mosquea Y.R.**, Kenysha Clear, Adam Wilson, Brian Westwood, David Soto-Pantoja, PhD Katherine Cook, PhD. Inositol-requiring enzyme-1 (IRE1) signaling impacts triple negative breast cancer chemotherapy sensitivity and prevents cardiac toxicity from chemotherapy. American Society for Matrix Biology (ASMB) Biennial Meeting in St. Louis, MO. September 12-15, 2021.
7. **Feliz-Mosquea Y.R.**, Manuel Ramirez, Kenysha Clear, Alaa Bawaneh, Adam Wilson, Nildris Cruz-Diaz, David R. Soto-Pantoja, Katherine L. Cook. Diet impacts triple negative breast cancer growth and the development of chemotherapy-induced cardiac damage. Second Annual Interdisciplinary Nutrition Sciences Symposium (INSS). June 21-22, 2021.
8. **Feliz-Mosquea Y.R.**, Kenysha Clear, Adam Wilson, Brian Westwood, David Soto-Pantoja, Katherine Cook. Blocking IRE1 signaling pathway sensitizes

- triple-negative breast tumors to chemotherapy and prevents cardiac toxicities. 11th Annual Women's Health Research Day. April 21, 2021.
9. **Feliz-Mosquea Y.R.**, Kenysha Clear, Adam Wilson, Brian Westwood, David Soto-Pantoja, Katherine Cook. Blocking IRE1 signaling pathway sensitizes triple-negative breast tumors to chemotherapy and prevents cardiac toxicities. The Graduate School of Arts and Sciences 2021 Virtual Research Day. April 12, 2021.
 10. **Feliz-Mosquea Y.R.**, Soto-Pantoja D.R, Clear K, Wilson A, Westwood B, and Cook K.L. Blocking UPR (IRE1) signaling impacts triple negative breast cancer chemotherapy sensitivity and prevents cardiac toxicity from chemotherapy. 28th Annual Surgical Sciences Residents' and Fellows' Research Day on November 19, 2020.
 11. **Feliz-Mosquea Y.R.**, Soto-Pantoja D.R, Clear K, Wilson A, Westwood B, and Cook K.L. Targeting the Unfolded Protein Response (UPR) prevents cardiac toxicity from chemotherapy. 27th Annual Surgical Sciences Residents' and Fellows' Research Day on November 7, 2019.
 12. **Feliz-Mosquea Y.R.**, Katherine L. Cook, Brian Westwood, Nancy J. Emenaker, David D. Roberts, David R. Soto-Pantoja. Thrombospondin-1 deletion in the Apc^{Min/+} mouse alters intestinal metabolism and microbiota during colon carcinogenesis. AICR 2019 Research Conference on Diet, Obesity, Physical Activity and Cancer, Chappell Hill, NC. May 15-17, 2019.
 13. **Feliz-Mosquea Y.R.**, Avery Salmon, Ana C. Melo, Adam Wilson, Jasmina Varagic, Giselle C. Melendez, Katherine L. Cook, David R. Soto-Pantoja. Anti-Cd47 Immunotherapy Prevents Chemotherapy-Related Cardiotoxicity by the Modulation of Mitochondrial Function. 26th Annual Surgical Sciences Residents' and Fellows' Research Day on November 7, 2018.
 14. **Feliz-Mosquea Y.R.**, E Stirling, KL Cook, A Wilson, M Bharadwaj, AJ Molina, L Yamaleyeva, PL Triozzi, DR Soto-Pantoja. *Anti- CD47 immunotherapy regulates T cell metabolism and hypoxia in the tumor microenvironment*. 2018 American Association for Cancer Research Annual (AACR) Meeting, Chicago Illinois. April 14-18, 2018.
 15. **Feliz-Mosquea Y.R.**, E Stirling, KL Cook, A Wilson, M Bharadwaj, AJ Molina, L Yamaleyeva, PL Triozzi, DR Soto-Pantoja. *Anti- CD47 immunotherapy regulates T cell metabolism and hypoxia in the tumor microenvironment*. 2018 Cancer & Immunotherapy Symposium in Washington DC. April 14, 2018.
 16. **Feliz-Mosquea Y.R.**, DR Soto-Pantoja, A Wilson, B Westwood, P Triozzi, and KL Cook. *Unfolded protein response signaling promotes T-cell dysfunction to prevent immune-mediated cancer cell killing and immune checkpoint therapy resistance*. 25th Annual Surgical Sciences Residents' and Fellows' Research Day at Wake Forest University School of Medicine on November 16, 2017.

17. **Feliz-Mosquea Y.R.**, DR Soto-Pantoja, A Wilson, B Westwood, P Triozzi, and KL Cook. *Unfolded protein response signaling promotes T-cell dysfunction to prevent immune-mediated cancer cell killing and immune checkpoint therapy resistance*. Dept of Surgery Symposium. Wake Forest University School of Medicine, Winston Salem, NC. November 8, 2017.
18. **Feliz-Mosquea Y.R.**, DR Soto-Pantoja, A Wilson, B Westwood, P Triozzi, and KL Cook. *Unfolded protein response signaling promotes T-cell dysfunction to prevent immune-mediated cancer cell killing and immune checkpoint therapy resistance*. 2017 Annual Biomedical Research Conference for Minority Students (ABRCMS) in Phoenix, AZ. November 1-4, 2017
19. **Feliz-Mosquea Y.R.**, David R. Soto-Pantoja, Adam Wilson, Pierre L. Triozzi, Katherine L. Cook. *UPR Signaling Promotes T-cell dysfunction to prevent immune-mediated cancer cell killing and immune checkpoint therapy resistance*. Center for Molecular Signaling. Fall Retreat, Thursday October 12, 2017; Wake Downtown, Winston Salem, NC.
20. **Feliz-Mosquea Y.R.**, David R. Soto-Pantoja, Adam Wilson, Pierre L. Triozzi, Katherine L. Cook. *UPR Signaling Promotes T-cell dysfunction to prevent immune-mediated cancer cell killing and immune checkpoint therapy resistance*. 2017 American Association for Cancer Research Annual (AACR) Meeting in Washington DC. April 1-5, 2017
21. **Feliz-Mosquea Y.R.**, David R. Soto-Pantoja, Adam Wilson, Pierre L. Triozzi, Katherine L. Cook. *UPR Signaling Promotes T-cell dysfunction to prevent immune-mediated cancer cell killing and immune checkpoint therapy resistance*. 2017 Cancer & Immunotherapy Symposium in Washington DC. March 31 to April 1, 2017.
22. **Feliz-Mosquea Y.R.**, Adam Wilson, Brian Westwood, Katherine L. Cook, Pierre L. Triozzi, David R. Soto-Pantoja. *Targeting CD47 Boosts Immune Cell Metabolism to Improve Responses to Cancer Immunotherapy*. The Twenty-Fourth Annual Surgical Residents' and Fellows' Research Day at Wake Forest University, Tuesday, November 8, 2016.
23. **Feliz-Mosquea Y.R.**, Cook K.L., Smith A., Soto-Pantoja D.R. *Synergistic Combinatorial Response of CD47 Blockade and Rapamycin in Breast Cancer*. 2016 SACNAS conference in Long Beach, CA. October 13-15, 2016.
24. **Feliz-Mosquea Y.R.**, Cook K.L., Smith A., Soto-Pantoja D.R. *Blockade of CD47 synergizes with rapamycin to overcome therapeutic resistant breast cancer*. Emory University-Laney Graduate School STEM Research and Career Symposium, Atlanta GA. September 18-20, 2016.
25. **Feliz-Mosquea Y.R.**, Suárez-Arroyo I., Leal-Alviárez D., Loperena Y., Cubano LA., Martínez-Montemayor MM. *Inhibitory effect of Ganoderma lucidum and lapatinib in HER2+ inflammatory breast cancer cells line*. 2015 ASCB Annual Meeting, San Diego, CA. December, 2015.

26. **Feliz-Mosquea Y.R.**, Suárez-Arroyo I., Leal-Alviárez D., Cubano LA., Martínez-Montemayor MM. *Improved Combinatorial Response of Ganoderma lucidum and the HER2 Tyrosine Kinase Inhibitor, Lapatinib in HER2+ Inflammatory Breast Cancer Cells*. Annual Biomedical Research Conference for Minority Students (ABRCMS), Washington State Convention Center in Seattle, WA. November, 2015.
27. **Feliz-Mosquea Y.R.**, Wang Wenqi., Chen Junjie. *Histone Deacetylase Inhibitors regulates the Hippo-YAP pathway in triple negative breast cancer cells*. The University of Texas MD Anderson Cancer Center, Houston, TX. August, 2015.
28. **Feliz-Mosquea Y.R.**, Suárez-Arroyo I., Loperena Y., Cubano LA., Martínez-Montemayor MM. *Enhancing response of Ganoderma lucidum (Reishi) and lapatinib in HER2+ inflammatory breast cancer cells*. AACR Annual Meeting 2015, Philadelphia PA. April, 2015.
29. **Feliz-Mosquea Y.R.**, Suárez-Arroyo I., Loperena Y., Cubano L.A., Martínez-Montemayor M.M. *Medicinal mushroom Reishi and lapatinib decrease the viability in HER2+ inflammatory breast cancer cells*. Emory University-Laney Graduate School STEM Research and Career Symposium, Atlanta GA. March, 2015.
30. **Feliz-Mosquea Y.R.**, Suárez-Arroyo I., Loperena Y., Cubano L.A., Martínez-Montemayor M.M. *Combination of the Medicinal Mushroom Reishi and the Tyrosine Kinase Inhibitor, Lapatinib inhibit the viability of Her2+ Inflammatory Breast Cancer Cell*. Annual Biomedical Research Conference for Minority Students (ABRCMS), San Antonio, TX. November, 2014.
31. **Feliz-Mosquea Y.R.**, Suárez-Arroyo I., Loperena Y., Cubano L.A., Martínez-Montemayor M.M. *Enhancing effect of Ganoderma lucidum (Reishi) and lapatinib against HER2+ inflammatory breast cancer cells*. 1st Puerto Rico Cancer Research Meeting, San Juan PR. October, 2014.
32. **Feliz-Mosquea Y.R.**, Suárez-Arroyo I., Loperena Y., Cubano L.A., Martínez-Montemayor M.M. *Reishi chemosensitizes IBC cells to lapatinib*. NIH, NIGMS 5th Biennial National IDeA Symposium of Biomedical Research Excellence (NISBRE), Washington D.C. June, 2014.
33. **Feliz-Mosquea Y.R.**, Suárez-Arroyo I., Loperena Y., Cubano L.A., Martínez-Montemayor M.M. *Synergistic effect between Ganoderma lucidum (Reishi) and lapatinib in HER2+ inflammatory breast cancer cells*. 12th Research Centers for Minority Institutions Cellular and Molecular Biology Center Annual Retreat sponsored by CMBC, NRC& SNRP Annual Retreat Guaynabo PR. May, 2014.
34. **Feliz-Mosquea Y.R.**, Suárez-Arroyo I., Cubano L.A., Martínez-Montemayor M.M. *Synergistic effect between Ganoderma lucidum (Reishi) and lapatinib in HER2+ inflammatory breast cancer cells*. AACR Annual Meeting and Ninth Annual AACR Undergraduate Student Caucus and Poster Competition, San Diego CA. April, 2014.

35. **Feliz-Mosquea Y.R.**, Loperena Y., Suárez-Arroyo I., Cubano L.A., Martínez-Montemayor M.M. *Ganoderma Lucidum (Reishi) Chemosensitizes Inflammatory Breast Cancer Cell Line, KPL-4 to lapatini*. 6th Annual Medical Students Association Convention, AEMPR, Dorado PR. March, 2014.
36. **Feliz-Mosquea Y.R.**, Rosario-Acevedo R., Suárez-Arroyo I., Cubano L.A., Martínez-Montemayor M.M. *Effect of Ganoderma lucidum (Reishi) in inflammatory breast cancer viability*. 37th ACS Senior Technical Meeting from American Chemical Society and INBRE, Aguadilla PR. November, 2013.
37. **Feliz-Mosquea Y.R.**, Martinez-Vaz B., Sadowski Michael. *1-aminocyclopropane-1-carboxylate (ACC) Deaminase Enzymes in Medicago-Nodulating Rhizobia*. The 13th Annual University of Minnesota Summer Undergraduate Research Symposium, Minneapolis MN. August, 2013.

FELLOWSHIPS AND AWARDS

1. **June, 2022** – T32 - NIH NRSA predoctoral fellowship in the Redox Biology and Medicine Training Program, grant T32GM127261 through NIGMS (period July 1, 2022 – June 30, 2023)
2. **June, 2022** - Travel Scholarship to attend the 2022 National Diversity in STEM (NDiSTEM) Conference
3. **May, 2022** - First Place Graduate Trainee Division WFSM Women's Health Research Day
4. **April, 2022** – Women's Health Research Day 2022 Top-10 Scoring abstracts
5. **November, 2021** – Wake Forest School of Medicine Surgical Sciences Residents' and Fellows' Research Day (Second Plate Poster Award)
6. **September, 2021** - GMaP Region 1 South Stimulus Award
7. **July, 2021** – URM Complimentary Registrations (Travel Award) for the ASMB Biennial Meeting
8. **June, 2021** - T32 - NIH NRSA Predoctoral Fellowship in the Redox Medicine and Biology Training Program grant 5T32GM127261-04 through NIGMS (period July 1, 2021 – June 30, 2022).
9. **May, 2021** - Interdisciplinary Nutrition Sciences Symposium Registration Sponsorship
10. **March, 2017** - ACEA Biosciences Inc. Travel Award to attend the 2017 Cancer & Immunotherapy Symposium in Washington DC. March 31 to April 1, 2017.
11. **May 2016** - Post-Baccalaureate Research Education Program (PREP) fellowship at Wake Forest Graduate School of Arts and Sciences in Winston-Salem, North Carolina (2016-2018).
12. **December 2015** - First Place in the Undergraduate category in the MAC poster competition at the ASCB Annual Meeting, in San Diego, C.A.
13. **November 2015** - Wake Forest University School of Medicine. 2016 Excellence in Cardiovascular Sciences (EICS) Summer Program fellowship.

14. **September, 2015** - American Society for Cell Biology (ASCB) - MAC Travel Award to attend the 2015 ASCB Meeting.
15. **May, 2015** - Academic Excellence Award. Program of Educational Services, Inter American University of Puerto Rico.
16. **February, 2015** - MD Anderson Cancer Center. CPRIT/CURE Summer Program fellowship.
17. **February, 2015** - Emory University-Laney Graduate School STEM Research and Career Symposium Full Travel Award.
18. **September, 2014** - Program for students of High Honor (PROGRESAH) Scholarships, Puerto Rico Department of Education (2014-2015).
19. **October, 2014** - Annual Biomedical Research Conference for Minority Students (ABRCMS), Travel Award.
20. **March, 2014** - Third place in the Molecular Biology category AEMPR during the 6th Annual Medical Students Association Convention, in Dorado, P.R.
21. **January 2014** - American Association for Cancer Research (AACR) - Thomas J. Bardos Science Education Award for Undergraduate Students (2014-2015).
22. **August, 2013** – Undergraduate Research Fellowship, awarded by NIH-NIGMS / Puerto Rico-Idea Network of Biomedical Research Excellence (INBRE) Program (2013-2014).
23. **May, 2013** - Academic Excellence Award, Inter American University of Puerto Rico.
24. **December 2012** - Part of Chancellor's (Dean's) List for Academic Excellence, Inter American University of Puerto Rico (2012-2016).
25. **August, 2012** - Fellowship for Academic Excellence, Honor Program, Inter American University of Puerto Rico (2012-2016).
26. **May, 2012** - Good Citizenship and Leadership Award for Community Services, Program of Educational Services, Inter American University of Puerto Rico.
27. **May, 2012** - Special distinction for my academic excellence and active participation in the Program of Educational Services at Inter American University of Puerto Rico.
28. **July, 2011** - Free Application for Federal Student Aid, FAFSA (2011-2016).
29. **May, 2011** - A distinction from the Puerto Rico Government for Academic Excellence.
30. **December, 2010** - Scholarship for my academic excellence from the Puerto Rico Department of education.

SKILLS

- **Language Skills:** Spanish and English.
- **Computer Skills:** PC and Mac Operating Systems, Microsoft Office, Graph Pad Prism and Cardiac Function (VEVO LAZR ultrasound system) analysis.
- **Microbiological techniques:** Transformation, isolation, extraction, ligation, miniprep, competent cells, streak and spread plates.
- **Cellular and Molecular techniques in eukaryotic cells:** Cell culture

techniques, cell viability and proliferation assays, DNA and RNA Extraction, cDNA synthesis and PCR, RT-PCR, Transfection, ACEA xCELLigence assay (Cytotoxicity), MST and LDH assays. Gel Electrophoresis (Agarose and Polyacrylamide), and Spectrophotometry. Light and Fluorescence microscopy.

- **Proteomic techniques:** Western blot, SDS PAGE, Protein quantification, Tandem Affinity Purification Mass Spectrometry (TAP-MS), SILAC and Seahorse.
- **Histological Techniques:** TUNEL staining, Musin staining, Immunohistochemistry/Immunofluorescence staining, DAB staining, Pic-red staining, Hematoxylin & eosin (H&E) and Trichrome staining.

PROFESSIONAL MEMBERSHIPS AND STUDENTS ORGANIZATION

- Student member of American Society for Matrix Biology (ASMB). **July 2021-present**
- Executive Committee Member of the NHLBI-Funded R25 Excellence in Cardiovascular Sciences
- Program (EICS)
- Student member of Radiation Biology /Department of Radiation Oncology at Wake Forest University School of Medicine. **2017-present**
- Student member of Society for Advancement of Chicanos and Native Americans in Science (SACNAS). **May 2016-present**
- Student member of American Society for Cell Biology (ASCB). **August 2015-present**
- Honor Student Organization “Tigres de Honor”, 2014-2016
- Vice-president for Sciences Students Association “Estudiantes Unidos por la Ciencias” (SEUC), 2014-2015
- Student member of American Association for Cancer Research (AACR). **2013-present**
- Student Support Services Student Community Service Association “Acción Comunitaria”- 2013-2015
- Student Community Service Association “Alianza Comunitaria” (ALICOM)- 2011- 2014
- Christian Student Organization “Hermandad de Universitarios Cristianos” (HUC), 2011- 2014

VOLUNTEER ACTIVITIES

2018 - Opioid Collection Day/Medication Take-Back Event-Volunteer, Wake Forest Baptist Health

2018 - Backpack Event, Wake Forest Baptist Medical Center – Volunteer, North Carolina

2018 - Serve the Lord with the youth ministry (leader and gives bible classes to children, etc.)-**Present**

2017 - Let's learn about your heart, Novozymes SciMatch Program- Volunteer, North Carolina

2015 - Good nutrition and academic achievement

2015 - Chemistry Festival- Volunteer, Puerto Rico

2014 - Children's home care, "Hogar de Niño Ave María" ("Acción Comunitaria") 2013 and 2014

2014 - "Visita al Santuario; Amor es Igual a Mascotas"

2014 - "Recogido de Artículos de primera necesidad; Dando Esperanza"

2014 - International Day of Beach Cleaning by Scuba Dogs Society- Volunteer, Puerto Rico

2013 - San Francisco de Asís Animals's Sanctuary- Volunteer, Puerto Rico, 2013-2014

2012 - Biology and Math Tutoring, Volunteer, Puerto Rico

2012 - President of Alianza Azarias (association that helps children and elderly people in and outside PR), **2012-present**

2012 - Operation Christmas Child- Volunteer, Puerto Rico, 2012-2015

2012 - Teens Home Care "Forjadores de Esperanza" (ALICOM), Puerto Rico

2012 - Children's home care, "Hogar del Niño Andrés" ("Alianza Comunitaria"), Puerto Rico

2011 - Community Lunch - Volunteer, Puerto Rico, 2011-2015

2009 - Professor Assistant, Volunteer, Dr. Agustín Stahl High School, Puerto Rico, 2009-2010

REFERENCES

David Soto Pantoja, Ph.D.

Assistant Professor

Department of Hypertension/Comprehensive Cancer Center/Maya Angelou
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Department of Natural Sciences and Mathematics

Inter American University of Puerto Rico, Bayamón Campus

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Ivette J. Suárez Arroyo, Ph.D.

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